(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 January 2001 (11.01.2001)

PCT

(10) International Publication Number
WO 01/02440 A1

(51) International Patent Classification[7]: C07K 19/00, C12N 15/62, 15/86, 5/10, C07K 14/705

(21) International Application Number: PCT/US00/18185

(22) International Filing Date: 30 June 2000 (30.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/142,088 2 July 1999 (02.07.1999) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: FUSION PEPTIDES COMPRISING A PEPTIDE LIGAND DOMAIN AND A MULTIMERIZATION DOMAIN

(57) Abstract: The invention provides novel hybrid molecules comprising a peptide ligand domain which functions to bind a predetermined target molecule and a multimerization domain such as an immunoglobulin constant region domain or a leucine zipper. The hybrid molecules optionally include an additional functional moiety such as an enzyme moiety or a cytotoxic moiety. In preferred embodiments, the hybrid molecules have improved pharmacokinetic or pharmacological properties. The invention further provides for the research, diagnostic and therapeutic use of the hybrid molecules and includes compositions such as pharmaceutical compositions comprising the hybrid molecules.

FUSION PEPTIDES COMPRISING A PEPTIDE LIGAND DOMAIN AND A MULTIMERIZATION DOMAIN

Background of the Invention

Field of the Invention

This invention relates to novel compositions comprising a peptide ligand domain which binds a predetermined target molecule and a multimerization domain such as an immunoglobulin constant region domain or a leucine zipper domain. The hybrid molecules optionally include an additional functional moiety such as an enzyme moiety or a cytotoxic moiety. In preferred embodiments, the hybrid molecules have improved pharmacokinetic or pharmacological properties. The invention further provides for the research, diagnostic and therapeutic use of the hybrid molecules and includes compositions such as pharmaceutical compositions comprising the hybrid molecules.

Description of Related Disclosures

Phage-display provides a means for generating constrained and unconstrained peptide libraries (Devlin et al., (1990) Science 249:404-406; Cwirla et al., (1990) Proc. Natl. Acad. Sci. USA 87:6378-6382; Lowman and Wells (1991) Methods: Comp. to Methods Enzymol. 3:205-216; Lowman (1997) Ann. Rev. Biophys. Biomol. Struct. 26:401-424). These libraries can be used to identify and select peptide ligands that can bind a predetermined target molecule (Lowman (1997), supra); Clackson and Wells (1994) Trends Biotechnol. 12:173-184; Devlin et al., (1990) supra). The technique has been used to identify peptide motifs that home to a cellular target (Arap et al., (1998) Science 279:377-380); bind the human type I interleukin 1 (IL-1) receptor blocking the binding of IL-1α (Yanofsky et al., (1996) Proc. Natl. Acad. Sci. USA, 93:7381-7386); bind to and activate the receptor for the cytokine erythropoietin (EPO)(Wrighton et al., (1996) Science 273:458-463); bind the human thrombopoietin receptor and compete with the binding of the natural ligand thrombopoietin (TPO)(Cwirla et al., (1996) Science, 276:1696-1699), or to generate affinity improved or matured peptide ligands from native protein binding ligands (Lowman et al., (1991) Biochemistry 30: 10832-10838).

Using structurally constrained peptide libraries generated by monovalent phage display, 14 amino acid peptides that specifically bind to insulin-like growth factor 1 binding proteins (IGFBPs) have been isolated (Lowman et al., (1998) Biochemistry, 37:8870-8878;. The peptides contain a helix structure and bind IGFBPs in vitro liberating insulin like growth factor-a (IGF-1) activity (Lowman et al., (1998) supra). Utilizing in vivo phage selection, the technique has been used to identify and isolate peptides capable of mediating selective localization to various organs such as brain and kidney (Pasqualini and Ruoslohti (1996) Nature 380:364-366) as well as to identify peptides that home to particular tumor types bearing $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins (Arap et al., (1998) Science 279:377-380). U.S.

Patent 5,627,263 describes peptides that are recognized by and selectively bind the $\alpha_5\beta_1$ integrin. Examples of affinity or specificity improved proteins include human growth hormone, zinc fingers, protease inhibitors, ANP, and antibodies (Wells, J. and Lowman H. (1992) Curr. Opin. Struct. Biol. 2:597-604; Clackson, T. and Wells, J. (1994) Trends Biotechnol. 12:173-184; Lowman et al., (1991) Biochemistry 30(10):832-838; Lowman et al. and Wells J. (1993) J. Mol. Biol. 234:564-578; Dennis M. and Lazarus R. (1994) J Biol. Chem. 269(22):137-144).

U.S. Patent No. 5,336,603 describes the fusion of an "adhesin" with a plasma protein such as an immunoglobulin. An adhesin is defined as a cell surface polypeptide having an extracellular domain homologous to a member of the immunoglobulin gene superfamily (excluding certain highly polymorphic members of the superfamily such as T cell antigen receptor α, β, γ and δ chains). According to that patent, an adhesin extracellular domain, or an immunoglobulin like domain of an adhesin extracellular domain, may be fused at its C-terminus to the N-terminus of an immunoglobulin constant region. Such a molecule is referred to therein as an immunoadhesin. Particular immunoadhesins include fusion proteins comprising at least one of the immunoglobulin like or variable (V) region domains of CD4 fused C-terminally to the N-terminus of an immunoglobulin heavy or light chain constant domain.

U.S. Patent 5,116,964 describes the fusion of a "ligand binding partner" to a stable plasma protein such as an immunoglobulin. Ligand binding partners are described as proteins known to function to bind specifically to target ligand molecules. The ligand binding partners are generally found in their native state as secreted and membrane bound polypeptides such as receptors, carrier proteins, hormones, cellular adhesive proteins, lectin binding molecules, growth factors, enzymes and nutrient substances. Truncated forms of a ligand binding partner, in which, for example, the transmembrane and cytoplasmic regions of membrane associated ligand binding partners have been deleted, fused to an immunoglobulin chain are also described.

Particular immunoadhesins have been described (Ashkenazi and Chamow (1997) Curr. Op. Immunol. 9:195-200; Chamow and Ashkenazi (1996) Trends Biotechnol. 14:52-60) and include CD4 (Capon et al., (1989) Nature 337:525-531; Traunecker et al., (1989) Nature 339:68-70; and Byrn et al., (1990) Nature 344:667-670); L-selectin or homing receptor (Watson et al., (1990) J. Cell. Biol. 110:2221-2229; and Watson et al., (1991) Nature 349:164-167); CD44 (Aruffo et al., (1990) Cell 61:1303-1313; CD28 and B7 (Linsley et al., (1991) J. Exp. Med. 173:721-730); CTLA-4 (Lisley et al., J. Exp. Med. 174:561-569); CD22 (Stamenkovic et al., Cell 66:1133-1144); TNF receptor (Ashkenazi et al., (1991) Proc. Natl. Acad. Sci. USA 88:10535-10539; Lesslauer et al., (1991) Eur. J. Immunol. 27:2883-2886; and Peppel et al., (1991) J. Exp. Med. 174:1483-1489); NP receptors (Bennett et al., (1991) J.

Biol. Chem. 266:23060-23067; interferon γ receptor (Kurschner et al., (1992) J. Biol. Chem. 267:9354-9360; 4-1BB (Chalupny et al., (1992) PNAS USA 89:10360-10364) and IgE receptor α (Ridgway and Gorman, (1991) J. Cell. Biol. 115, Abstract No. 1448).

Others have described a short peptide ligand fused via a semi-rigid hinge region from camel IgG with the coiled-coil assembly domain of the cartilage oligomeric matrix protein (Efimov et al., (1994) FEBS Lett. 341:54-58; Tomschy et al., (1996) EMBO J. 15:3507-3514) expressed as a stable homopentamer in E. coli (Terskikh et al., (1997) Proc. Natl. Acad. Sci. USA 94:1663-1668).

Summary of the Invention

The present invention provides novel compositions comprising a peptide ligand domain which binds to a predetermined target molecule and a multimerization domain such as an immunoglobulin constant region domain or a leucine zipper domain. The hybrid molecules optionally include an additional functional moiety such as an enzyme moiety or a cytotoxic moiety. In preferred embodiments, the hybrid molecules comprising a peptide ligand domain have improved pharmacokinetic or pharmacological properties providing low dose pharmaceutical formulations and novel pharmaceutical compositions. In certain aspects, the invention provides for methods of using the novel compositions including the therapeutic use of the hybrid molecules.

Accordingly, the invention provides a polypeptide amino acid sequence which comprises a peptide ligand domain amino acid sequence and a multimerization domain sequence. The peptide ligand is preferably a non-naturally occurring amino acid sequence that binds a predetermined target molecule. Preferably the peptide ligand is a non-naturally occurring constrained or unconstrained amino acid sequence of between about 3 and about 50 amino acid residues and preferably between about 10 and about 40 amino acid residues and more preferably the peptide ligand is between about 20 and about 30 amino acid residues.

According to the present invention the peptide ligand domain sequence is linked to a multimerization domain, optionally via a flexible peptide linker. The multimerization domain is preferably an immunoglobulin sequence or, for example, a leucine zipper sequence. According to this aspect of the invention, the immunoglobulin sequence is preferably an immunoglobulin constant region sequence and especially the constant region of an immunoglobulin heavy chain. According to preferred aspects of the invention, the multimerization domain pairs with one or more companion multimerization domains to provide homo- and hetero-multimer compositions. According to this aspect of the present invention, provided are homo- and hetero-multimers, especially homo- and heterodimers wherein the multimerization domains are immunoglobulin heavy chain constant regions which pair to provide functional immunoglobulin Fc domains. Therefore,

according to certain aspects, the invention provides a hybrid molecule comprising at least one peptide ligand domain sequence which functions to target the hybrid molecule to a predetermined target molecule such as a specific cell type and functional immunoglobulin Fc domain possessing an effector function associated with a functional immunoglobulin Fc domain.

In one embodiment, the composition of the present invention is a polypeptide and the invention encompasses a composition of matter comprising an isolated nucleic acid, preferably DNA, encoding the polypeptide of the invention. According to this aspect, the invention further comprises an expression control sequence operably linked to the DNA molecule, an expression vector, preferably a plasmid, comprising the DNA molecule, where the control sequence is recognized by a host cell transformed with the vector, and a host cell transformed with the vector. According to preferred aspects the nucleic acid encodes a hybrid molecule comprising a peptide ligand domain sequence and an immunoglobulin constant region domain sequence. The nucleic acid molecule according to this aspect of the present invention encodes a hybrid molecule and the nucleic acid encoding the peptide ligand domain sequence is operably linked to (in the sense that the DNA sequences are contiguous and in reading frame) the immunoglobulin domain sequence. Optionally the DNA sequences may be linked through a nucleic acid sequence encoding an optional linker domain amino acid sequence.

The compositions of the present invention may be made by a process which includes the steps of isolating or synthesizing nucleic acid sequences encoding any of the amino acid sequences of the invention, ligating the nucleic acid sequence into a suitable expression vector capable of expressing the nucleic acid sequence in a suitable host, transforming the host with the expression vector into which the nucleic acid sequence has been ligated, and culturing the host under conditions suitable for expression of the nucleic acid sequence, whereby the protein encoded by the selected nucleic acid sequence is expressed by the host. Preferably, the polypeptide is then recovered from the host cell culture. In this process, the ligating step may further contemplate ligating the nucleic acid into a suitable expression vector such that the nucleic acid is operably linked to a suitable secretory signal, whereby the amino acid sequence is secreted by the host.

The invention further provides for an optional functional domain attached to the hybrid molecule. The optional functional domain provides an additional function to the hybrid molecule such as the function associated with an enzyme. For example, the additional functional domain may be an enzyme, covalently linked to the hybrid molecule and capable of acting on a prodrug in such a way as to convert the prodrug to its more active form. The optional functional domain, according to certain preferred aspects of the invention, may be a cytotoxic agent linked by, for example, covalent

attachment, to the hybrid molecule. Preferred cytotoxic agents include, for example, chemotherapeutic agents, toxins and radioactive isotopes.

The present invention further extends to therapeutic and diagnostic applications for the compositions described herein. Therefore, the invention includes pharmaceutical compositions comprising a pharmaceutically acceptable excipient and the hybrid molecules of the invention.

Brief Description of the Drawings

Figure 1 is a schematic representation of a native IgG and fragments thereof resulting from papain and pepsin digest. Disulfide bonds are represented by -S-S- between, for example, the CH1 and CL domains. In the figure, V is variable domain; C is constant domain; L stands for light chain and H stands for heavy chain.

Figure 2A depicts alignments of native IgG Fc region amino acid sequences. Shown are human (hum) IgG Fc region sequences, humIgG1 (non-A and A allotypes) (SEQ ID NOs: 1 and 2, respectively), humIgG2 (SEQ ID NO:3), humIgG3 (SEQ ID NO:4) and humIgG4 (SEQ ID NO:5), are shown. The human IgG1 sequence is the non-A allotype, and differences between this sequence and the A allotype (at positions 356 and 358; EU numbering system) are shown below the human IgG1 sequence. Also shown are native murine (mur) IgG Fc region sequences, murIgG1 (SEQ ID NO:6), murIgG2A (SEQ ID NO:7), murIgG2B (SEQ ID NO:8) and murIgG3 (SEQ ID NO:9), are also shown. Figure 2B shows the percent identity among the Fc region sequences of Figure 2A.

Figure 3 shows the alignments of native human IgG Fc region sequences, humIgG1 (non-A and A allotypes), humIgG2, humIgG3 and humIgG4 with differences between the sequences marked with asterisks.

Figure 4 shows SDS-PAGE analysis of the reduced and unreduced TF151-Fc and HER2-Fc revealing bands at about 30 and about 60 kDa suggesting the association of two peptide-Fc monomers to form a dimer.

Figure 5 shows the results of an ELISA to determine the ability of TF151-Fc to compete with TF147b (SEQ ID NO:10) for binding to FVIIa. The ability of TF151-Fc to compete against TF147b for binding to FVIIa was comparable to the ability of the peptide ligand, TF151 alone.

Figure 6 shows the ability of the TF151-Fc fusion to inhibit FX activation by TF/FVIIa in a FX activation assay; control-Fc and HER2-Fc had no effect in this assay.

Figure 7 TF151-Fc prolongs the prothrombin time (PT) in human plasma.

Figure 8 Using a HER2 phage binding ELISA assay the HER2-Fc is able to block 1.1F1 phage binding to immobilized HER2-ECD with an IC50 of 3 nM.

Figure 9 HER2-Fc has an IC50 similar to peptide 1.1FI-Z in a HER2 competition ELISA.

Detailed Description of the Preferred Embodiments

Definitions

The term "peptide ligand" within the context of the present invention is meant to refer to non-naturally occurring amino acid sequences that function to bind a particular target molecule. Peptide ligands within the context of the present invention are generally constrained (that is, having some element of structure as, for example, the presence of amino acids which initiate a β turn or β pleated sheet, or for example, cyclized by the presence of disulfide bonded Cys residues) or unconstrained (linear) amino acid sequences of less than about 50 amino acid residues, and preferably less than about 40 amino acids residues. Of the peptide ligands less than about 40 amino acid residues, preferred are the peptide ligands of between about 10 and about 30 amino acid residues and especially the peptide ligands of about 20 amino acid residues. However, upon reading the instant disclosure, the skilled artisan will recognize that it is not the length of a particular peptide ligand but its ability to bind a particular target molecule that distinguishes the peptide ligand of the present invention. Therefore peptide ligands of 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 and 25 amino acid residues, for example, are equally likely to be peptide ligands within the context of the present invention.

A peptide ligand of the present invention will bind a target molecule with sufficient affinity and specificity if the peptide ligand "homes" to, "binds" or "targets" a target molecule such as a specific cell type bearing the target molecule in vitro and preferably in vivo (see, for example, the use of the term "homes to," "homing," and "targets" in Pasqualini and Ruoslahti (1996) Nature, 380:364-366 and Arap et al., (1998) Science 279:377-380). In general, the peptide ligand will bind a target molecule with an affinity of less than about 1 μM, preferably less about 100 nM and more preferably less than about 10 nM. However, peptide ligands having an affinity for a target molecule of less than about 1 nM and preferably between about 1 pM and 1 nM are equally likely to be peptide ligands within the context of the present invention. In general a peptide ligand that binds a particular target molecule as described above can be isolated and identified by any of a number of art standard techniques as described herein.

Peptides ligands are amino acid sequences as described above which may contain naturally as well as non-naturally occurring amino acid residues. Therefore, so-called "peptide mimetics" and "peptide analogs" which may include non-amino acid chemical structures that mimic the structure of a particular amino acid or peptide may be peptide ligands within the context of the invention. Such mimetics or analogs are characterized generally as exhibiting similar physical characteristics such as size, charge or

hydrophobicity present in the appropriate spacial orientation as found in their peptide counterparts. A specific example of a peptide mimetic compound is a compound in which the amide bond between one or more of the amino acids is replaced by, for example, a carbon-carbon bond or other bond as is well known in the art (see, for example Sawyer, in Peptide Based Drug Design pp. 378-422 (ACS, Washington DC 1995).

Therefore, the term "amino acid" within the scope of the present invention is used in its broadest sense and is meant to include naturally occurring L α-amino acids or residues. The commonly used one and three letter abbreviations for naturally occurring amino acids are used herein (Lehninger, A.L., Biochemistry, 2d ed., pp. 71-92, (1975), Worth Publishers, New York). The term includes D-amino acids as well as chemically modified amino acids such as amino acid analogs, naturally occurring amino acids that are not usually incorporated into proteins such as norleucine, and chemically synthesized compounds having properties known in the art to be characteristic of an amino acid. For example, analogs or mimetics of phenylalanine or proline, which allow the same conformational restriction of the peptide compounds as natural Phe or Pro are included within the definition of amino acid. Such analogs and mimetics are referred to herein as "functional equivalents" of an amino acid. Other examples of amino acids are listed by Roberts and Vellaccio (The Peptides: Analysis, Synthesis, Biology,) Eds. Gross and Meiehofer, Vol. 5 p 341, Academic Press, Inc, N.Y. 1983, which is incorporated herein by reference.

Peptide ligands synthesized by, for example, standard solid phase synthesis techniques, are not limited to amino acids encoded by genes. Commonly encountered amino acids which are not encoded by the genetic code, include, for example, those described in International Publication No. WO 90/01940 such as, for example, 2-amino adipic acid (Aad) for Glu and Asp; 2-aminopimelic acid (Apm) for Glu and Asp; 2-aminobutyric (Abu) acid for Met, Leu, and other aliphatic amino acids; 2-aminoheptanoic acid (Ahe) for Met, Leu and other aliphatic amino acids; 2-aminoisobutyric acid (Aib) for Gly; cyclohexylalanine (Cha) for Val, and Leu and Ile; homoarginine (Har) for Arg and Lys; 2,3-diaminopropionic acid (Dpr) for Lys, Arg and His; N-ethylglycine (EtGly) for Gly, Pro, and Ala; N-ethylglycine (EtGly) for Gly, Pro, and Ala; N-ethylasparigine (EtAsn) for Asn, and Gln; Hydroxyllysine (Hyl) for Lys; allohydroxyllysine (AHyl) for Lys; 3-(and 4)hydoxyproline (3Hyp, 4Hyp) for Pro, Ser, and Thr; allo-isoleucine (AIle) for Ile, Leu, and Val; p-amidinophenylalanine for Ala; N-methylglycine (MeGly, sarcosine) for Gly, Pro, and Ala; N-methylisoleucine (MeIle) for Ile; Norvaline (Nva) for Met and other aliphatic amino acids; Norleucine (Nle) for Met and other aliphatic amino acids; Ornithine (Orn) for Lys, Arg and His; Citrulline (Cit) and methionine sulfoxide (MSO) for Thr, Asn and Gln;

-methylphenylalanine (MePhe), trimethylphenylalanine, halo (F, Cl, Br, and I)phenylalanine, triflourylphenylalanine, for Phe.

Peptide ligands within the context of the present invention are non-native or non-naturally occurring peptide ligands. By non-native is meant that the amino acid sequence of the particular peptide ligand is not found in nature. That is to say, amino acid sequences of non-native peptide ligands do not correspond to an amino acid sequence of a naturally occurring protein or polypeptide nor are the non-naturally occurring amino acid sequences "derived from" a naturally occurring amino acid sequence in the sense that they differ from a naturally occurring amino acid sequence by site directed addition, substitution or deletion of a limited number of amino acids. Peptide ligands of this variety may be produced or selected using a variety of techniques well known to the skilled artisan. For example, constrained or unconstrained peptide libraries may be randomly generated and displayed on phage utilizing art standard techniques, for example, Lowman et al., (1998) Biochemistry 37:8870-8878.

At least three distinct species of peptide ligands can be distinguished based upon function associated with binding a particular target molecule. They will be referred to herein as "neutral," "agonist" and "antagonist" peptide ligands. In general, a neutral peptide ligand functions to bind a particular target molecule as described above. Neutral peptide ligands are preferred in aspects of the present invention where targeting of a particular cell type bearing a target molecule with, for example, a cytotoxic agent or an enzyme is desired. An "agonist" peptide ligand, in addition to binding a predetermined target molecule has a direct effect on its target molecule. Agonist peptide ligands bind, for example, a particular cellular receptor, and preferably, will initiate a reaction or activity associated with the binding of the native ligand to the receptor. As but another example, an agonist peptide ligand may bind a particular cellular target molecule and initiate an activation event such as a phosphorylation event. By contrast, an "antagonist" peptide ligand acts to reduce or inhibit the activity, for example, the response induced by the native ligand, by binding to and, without limitation, in certain embodiments, blocking the association of the target molecule with the native or naturally occurring ligand.

Therefore, in preferred embodiments, peptide ligands are size restricted, constrained or unconstrained, non-naturally occurring amino acids sequences, optionally having either neutral, agonist or antagonist activity associated with their binding to a particular target molecule. Preferred peptide ligands are non-naturally occurring amino acid sequences of between about 5 and about 40 amino acids, more preferably between about 10 and about 30 amino acids and preferably between about 15 and 25 amino acids.

Examples of preferred peptide ligands include, for example, those described in the Example sections herein, as well as, for example:

i) peptides capable of mediating selective localization to various organs such as brain and kidney (Pasqualini and Ruoslohti (1996) Nature 380:364-366). Often these peptides contain dominant amino acid motifs such as the Ser-Arg-Leu motif found in peptides localizing to brain (Pasqualini and Ruoslahti (1996) supra).

ii) peptides containing amino acid sequences recognizing structurally related receptors such as integrins. For example, the amino acid sequence Arg-Gly-Asp (RGD) is found in extracellular matrix proteins such as fibrinogen, fibronectin, von Willibrand Factor and thrombospondin that are known to bind various integrins found on platelets, endothelial cells leukocytes, lymphocytes, monocytes and granulocytes. Peptides containing the RGD motif can be used to modulate the activity of the RGD recognizing integrins (Gurrath et al., (1992) Eur. J. Biochem. 210:911-921; Koivunen et al., (1995) Bio/Technology 13:265-270; O'Neil et al., (1992) Proteins 14:509-515). For example, peptides capable of homing specifically to tumor blood vessels such as those identified by in vivo phage selection contain the Arg-Gly-Asp (RGD) motif embedded in the peptide structure and binds selectively to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins(Arap et al., (1998) Science 279:377-380).

iii) phage display of peptide libraries has yielded short peptides with well defined solution conformation that can bind, for example, insulin like growth factor binding protein-1 and produce insulin growth factor like activity (Lowman et al., (1998) Biochemistry 37:8870-8878.

iv) small peptides isolated by random phage display of peptide libraries which bind to and activate the cellular receptors such as the receptor for EPO, optionally including full agonist peptides such as those which stimulate erythropoiesis described by Wrighton et al., (1996) Science 273:458-463; or those that stimulate proliferation of TPO responsive cells and described by Cwirla et al., (1997) Science 276:1696-1699).

v) peptides capable of blocking the interaction between a cytokine and its receptor such as the high affinity type I IL-1 receptor antagonists described by Yanofsky et al., (1996) Proc. Natl. Acad. Sci. USA 93:7381-7386.

Additional exemplary peptide epitopes are listed in Table I of Lowman (1997) Annu. Rev. Biophys. Biomol. Struct. 26:401-424 and described in the references cited therein the disclosures of which are hereby incorporated by reference.

As used within the context of the present invention the term "target molecule" includes, proteins, peptides, glycoproteins, glycopeptides, glycolipids, polysaccharides, oligosaccharides, nucleic acids, and the like. Target molecules include, for example, extracellular molecules such as

various serum factors including but not limited to serum proteins, the proteins involved in the complement cascade and serine proteases, including the factors involved in the coagulation cascade. Target molecules according to the present invention also include cell associated target molecules such as cellular receptors or cellular distribution (CD) antigens expressed on particular cell types, and include, for example:

i) organ selective address molecules on endothelial cell surfaces such as those which have been identified for lymphocyte homing to various lymphoid organs and to tissues undergoing inflammation (Belivaqua, et al (1989) Science, 243:1160-1165; Siegelman et al., (1989) Science 243:1165-1171; Cepek et al. (1994) Nature 372:190-193 and Rosen and Bertozzi (1994) Curr. Opin. Cell Biol. 6:663-673).

ii) endothelial cell markers responsible for tumor homing to various organs such as lungs (Johnson et al., (1993) J. Cell. Biol. 121:1423-1432).

iii) tumor cell antigens or "tumor antigens" that serve as markers for the presence of a preneoplastic or a neoplastic cell. Tumor antigens are classified into two broad categories: tumor-specific antigens (TSA) and tumor-associated antigens (TAA). See, for example, Klein, J., 1990, *Immunology*, Blackwell Scientific Publications, Inc., Cambridge, MA, pp. 419-428.

The term "multimerization domain" as used in the context of the present invention, is meant to refer to the portion of the molecule to which the peptide ligand is joined, either directly or through a "linker domain." The multimerization domain is preferably a polypeptide domain which, according to preferred embodiments, facilitates the interaction of two or more multimerization domains. While the multimerization domain promotes the interaction between two or more multimerization domains, there is no requirement within the context of the present invention that the peptide ligand joined to a multimerization domain be present as a portion of a multimer.

According to preferred aspects of the present invention, the multimerization domain is a polypeptide which promotes the stable interaction of two or more multimerization domains. By way of example and not limitation, a multimerization domain may be an immunoglobulin sequence, such as an immunoglobulin constant region, a leucine zipper, a hydrophobic region, a hydrophilic region, a polypeptide comprising a free thiol which forms an intermolecular disulfide bond between two or more multimerization domains or, for example a "protuberance-into-cavity" domain described in U.S. Patent 5,731,168. In that patent, protuberances are constructed by replacing small amino acid side chains from the interface of a first polypeptide with a larger side chain (for example a tyrosine or tryptophan). Compensatory cavities of identical or similar size to the protuberances are optionally created on the interface of a second polypeptide by replacing

large amino acid side chains with smaller ones (for example alanine or threonine).

Therefore, in a preferred aspect, the multimerization domain provides that portion of the molecule which promotes or allows the formation of dimers from monomeric multimerization domains. Preferably, according to this aspect of the invention, multimerization domains are immunoglobulin constant region domains. Immunoglobulin constant domains provide the advantage of improving in vivo circulating half-life of the compounds of the invention and optionally allow the skilled artisan to incorporate an "effector function" as described herein below into certain aspects of the invention.

Throughout the present specification and claims, the numbering of the residues in an immunoglobulin heavy chain is that of the EU index as in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991), expressly incorporated herein by reference. The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody.

"Antibodies" (Abs) and "immunoglobulins" (Igs) are proteins, generally glycoproteins, having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Antibodies" and "immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 Daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has an amino (N) terminal variable domain (VH) followed by carboxy (C) terminal constant domains. Each light chain has a variable N-terminal domain (VL) and a C-terminal constant domain; the constant domain of the light chain (CL) is aligned with the first constant domain (CH1) of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. According to the domain definition of immunoglobulin polypeptide chains, light (L) chains have two conformationally similar domains VL and CL; and heavy chains have four domains (VH, CH1, CH2, and CH3) each of which has one intrachain disulfide bridge.

Depending on the amino acid sequence of the constant (C) domain of the heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM. The immunoglobulin class can be further divided into subclasses (isotypes),

e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are α, δ, ε, γ, and μ domains respectively. The light chains of antibodies from any vertebrate species can be assigned to one of two distinct types called kappa (κ) or lambda (λ), based upon the amino acid sequence of their constant domains. Sequence studies have shown that the μ chain of IgM contains five domains VH, CHμ1, CHμ2, CHμ3, and CHμ4. The heavy chain of IgE (ε) also contains five domains.

The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known. Of these IgA and IgM are polymeric and each subunit contains two light and two heavy chains. The heavy chain of IgG (γ) contains a length of polypeptide chain lying between the CHγ1 and CHγ2 domains known as the hinge region. The α chain of IgA has a hinge region containing an O-linked glycosylation site and the μ and ε chains do not have a sequence analogous to the hinge region of the γ and α chains, however, they contain a fourth constant domain lacking in the others. The domain composition of immunoglobulin chains can be summarized as follows:

Light Chain λ = Vλ Cλ
κ = Vκ Cκ

Heavy Chain IgG (γ) = VH CHγ1, hinge CHγ2 CHγ3
IgM (μ) = VH CHμ1 CHμ2 CHμ3 CHμ4
IgA (α) = VH CHα1 hinge CHα2 CHα3
IgE (ε) = VH CHε1 CHε2 CHε3 CHε4
IgD (δ) = VH CHδ1 hinge CHδ2 CHδ3

The "CH2 domain" of a human IgG Fc region (also referred to as "Cγ2" domain) usually extends from about amino acid 231 to about amino acid 340. The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule.

The "CH3 domain" comprises the stretch of residues C-terminal to a CH2 domain in an Fc region (*i.e.* from about amino acid residue 341 to about amino acid residue 447 of an IgG).

"Hinge region" is generally defined as stretching from Glu216 to Pro230 of human IgG1 (Burton, *Molec. Immunol.*22:161-206 (1985)). Hinge regions of other IgG isotypes may be aligned with the IgG1 sequence by placing the first and last cysteine residues forming inter-heavy chain S-S bonds in the same positions.

The "lower hinge region" of an Fc region is normally defined as the stretch of residues immediately C-terminal to the hinge region, *i.e.* residues 233 to 239 of the Fc region.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments or regions, each with a single antigen-binding site, and a residual "Fc" fragment or region (Figure 1). Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The Fc region of an immunoglobulin generally comprises two constant domains, CH2 and CH3, as shown, for example, in Fig. 1. A "native Fc region sequence" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native human Fc region sequences are shown in Fig. 2 & 3 and include but are not limited to the human IgG1 Fc region (non-A and A allotypes); human IgG2 Fc region; human IgG3 Fc region; and human IgG4 Fc region as well as naturally occurring variants thereof. Native murine Fc regions sequences are shown in Fig. 2A.

Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen. The Fab fragment contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

A "functional Fc region" possesses an "effector function" of a native Fc region. Exemplary "effector functions" include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (*e.g.* B cell receptor; BCR), etc. Such effector functions generally require the Fc region to be combined with a binding domain (*i.e*, a peptide ligand herein) and can be assessed using various assays known in the art.

The effector functions mediated by the antibody Fc region can be divided into two categories: (1) effector functions that operate after the binding of antibody to an antigen (these functions involve the participation of the complement cascade or Fc receptor (FcR)-bearing cells); and (2) effector functions that operate independently of antigen binding (these functions confer persistence in the circulation and the ability to be transferred across cellular barriers by transcytosis). Ward and Ghetie, (1995) Therapeutic Immunology 2:77-94.

By introducing the appropriate amino acid sequence modifications in a parent or native Fc region, one can generate a variant Fc region which (a) mediates antibody-dependent cell-mediated cytotoxicity (ADCC) in the presence of human effector cells more or less effectively and/or (b) binds an Fc gamma receptor (FcγR) with greater or lesser affinity than the parent polypeptide. Such Fc region variants will generally comprise at least one amino acid modification in the Fc region. The variant Fc region may include two, three, four, five, etc substitutions therein (U.S. Patent No. 5,648,260 issued on July 15, 1997, and U.S. Patent No. 5,624,821 issued on April 29, 1997).

Several antibody effector functions are mediated by Fc receptors (FcRs), which bind the Fc region of an antibody. FcRs are defined by their specificity for immunoglobulin isotypes; Fc receptors for IgG antibodies are referred to as FcγR, for IgE as FcεR, for IgA as FcαR and so on. Three subclasses of FcγR have been identified: FcγR I (CD64), FcγR II (CD32) and FcγR III (CD16). Because each FcγR subclass is encoded by two or three genes, and alternative RNA spicing leads to multiple transcripts, a broad diversity in FcγR isoforms exists. These different FcR subtypes are expressed on different cell types (reviewed in Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-492 (1991)). For example, in humans, FcγRIIIB is found only on neutrophils, whereas FcγRIIIA is found on macrophages, monocytes, natural killer (NK) cells, and a subpopulation of T-cells. Notably, FcγRIIIA is the only FcR present on NK cells, one of the cell types implicated in ADCC.

The binding site on human and murine antibodies for FcγR have been previously mapped to the lower hinge region (residues 233-239: EU index numbering as in Kabat *et al.*, Sequences *of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). Woof *et al. Molec. Immunol.* 23:319-330 (1986); Duncan *et al. Nature* 332:563 (1988); Canfield and Morrison, *J. Exp. Med.* 173:1483-1491 (1991); Chappel *et al., Proc. Natl. Acad. Sci USA* 88:9036-9040 (1991).

"C1q" is a polypeptide that includes a binding site for the Fc region of an immunoglobulin. C1q together with two serine proteases, C1r and C1s, forms the complex C1, the first component of the complement dependent cytotoxicity (CDC) pathway.

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express FcRs (*e.g.* Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells in summarized is Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991).

As used herein, the term "salvage receptor binding ligand" refers to an ligand of the Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the compositions comprising the peptide ligands of the invention. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question.

Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

As used herein, the term "pulmonary administration" refers to administration of a formulation of the invention through the lungs by inhalation. As used herein, the term "inhalation" refers to intake of air to the alveoli. In specific examples, intake can occur by self-administration of a formulation of the invention while inhaling, or by administration via a respirator, e.g., to an patient on a respirator. The term "inhalation" used with respect to a formulation of the invention is synonymous with "pulmonary administration."

As used herein, the term "parenteral" refers to introduction of a compound of the invention into the body by other than the intestines, and in particular, intravenous (i.v.), intraarterial (i.a.), intraperitoneal

(i.p.), intramuscular (i.m.), intraventricular, and subcutaneous (s.c.) routes.

As used herein, the term "aerosol" refers to suspension in the air. In particular, aerosol refers to the particlization of a formulation of the invention and its suspension in the air. According to the present invention, an aerosol formulation is a formulation comprising a compound of the present invention that is suitable for aerosolization, i.e., particlization and suspension in the air, for inhalation or pulmonary administration.

Modes for Carrying out the Invention

The hybrid molecules of the present invention comprise at least two distinct domains. Each molecule of the present invention contains a peptide ligand domain and a multimerization domain. According to the present invention the peptide ligand domain is joined to a multimerization domain such as an immunoglobulin Fc region, optionally via a flexible amino acid linker domain.

Peptide Ligand Combinations

A. Multimerization domains

The hybrid molecules of the present invention are constructed by combining the peptide ligands with a suitable multimerization domain. Depending on the type of linkage and its method of production the peptide ligand may be joined via its - or C-terminus to the - or C-terminus of multimerization domain. For example, when preparing the hybrid molecules of the present invention via recombinant techniques, nucleic acid encoding a peptide ligand will be operably linked to nucleic acid encoding the multimerization domain sequence, optionally via a linker domain. Typically the construct encodes a fusion protein wherein the C-terminus of the peptide ligand is joined to the N-terminus of the multimerization domain. However, especially when synthetic techniques are employed, fusions where, for example, the N-terminus of the peptide ligand is joined to the N or C-terminus of the multimerization domain are also possible.

Preferred multimerization domains are immunoglobulin constant region sequences. Typically, in such fusions the encoded hybrid molecule will retain at least hinge, CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made, for example, to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain.

The precise amino acid site at which the fusion of the peptide ligand to the immunoglobulin constant domain is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion, or binding characteristics. In this regard, the skilled artisan may reference the construction of various immunoadhesins

described in the literature (U.S. Patent Nos. 5,116,964, 5,714,147 and 5,336,603; Capon et al., (1989) Nature 337:525-531; Traunecker et al., (1989) Nature 339:68-70; and Byrn et al., (1990) Nature 344:667-670; Watson et al., (1990) J. Cell. Biol. 110:2221-2229; Watson et al., (1991) Nature 349:164-167; Aruffo et al., (1990) Cell 61:1303-1313; Linsley et al., (1991) J. Exp. Med. 173:721-730; Lisley et al., J. Exp. Med. 174:561-569; Stamenkovic et al., Cell 66:1133-1144; Ashkenazi et al., (1991) Proc. Natl. Acad. Sci. USA 88:10535-10539; Lesslauer et al., (1991) Eur. J. Immunol. 27:2883-2886; and Peppel et al., (1991) J. Exp. Med. 174:1483-1489; Mahler et al., (1993) J. Immunol. 151:1548-1561); Bennett et al., (1991) J. Biol. Chem. 266:23060-23067; Kurschner et al., (1992) J. Biol. Chem. 267:9354-9360; Chalupny et al., (1992) PNAS USA 89:10360-10364; Ridgway and Gorman, (1991) J. Cell. Biol. 115, Abstract No. 1448).

According to a particular aspect, an immunoglobulin type multimerization domain is selected to provide a multimer such as a dimer having a functional Fc. The multimerization domain is selected to provide an Fc domain having an effector function associated with a native immunoglobulin Fc region. Therefore, the peptide ligand is joined, in particular aspects, to an immunoglobulin heavy chain constant domain to provide a multimer comprising a functional Fc domain selected for a particular effector function or functions. In this case, DNA encoding an immunoglobulin chain-peptide ligand sequence is typically coexpressed with the DNA encoding a second peptide ligand-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chains. The skilled artisan will recognize that effector functions include, for example, C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; and down regulation of cell surface receptors (*e.g.* B cell receptor; BCR) and prolonging half-life through incorporation of the salvage receptor binding ligand as described in, for example, U.S. patent no. 5,739,277 issued April 14, 1998.

Preferably, the Fc region is a human Fc region, e.g. a native sequence human Fc region such as a human IgG1 (A and non-A allotypes), IgG2, IgG3 or IgG4 Fc region. Such sequences are shown in Figures 2 & 3.

Additionally, by introducing the appropriate amino acid sequence modifications in a parent Fc region, one can generate a variant Fc region which (a) mediates antibody-dependent cell-mediated cytotoxicity (ADCC) in the presence of human effector cells more or less effectively and/or (b) binds an Fc gamma receptor (Fc(R) with greater or lesser affinity than the native sequence. Such Fc region variants will generally comprise at least one amino acid modification in the Fc region.

In a particular embodiment, the peptide ligand sequence is fused to the N-terminus of the Fc region of immunoglobulin G and in certain aspects to IgG1. It is possible to fuse the entire heavy chain constant region to the peptide ligand sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG Fc chemically (*i.e.* residue 216, taking the first residue of heavy chain constant region to be 114), or analogous sites of other immunoglobulins is used in the fusion. In a particularly preferred embodiment, the peptide ligand amino acid sequence is fused to (a) the hinge region and CH2 and CH3 or (b) the CH1, hinge, CH2 and CH3 domains, of an IgG heavy chain.

According to a particular aspect of this embodiment, hybrid molecules comprising a peptide ligand and a multimerization domain are assembled as multimers, for example homodimers, or heterodimers or even heterotetramers. Homodimers result from the pairing or crosslinking of two monomers comprising a peptide ligand and a multimerization domain. However, it is not essential that two identical monomers pair. According to a particular aspect of the invention a hybrid molecule as defined herein comprising a peptide ligand and a multimerization domain such as an immunoglobulin constant domain may pair with a companion immunoglobulin chain comprising one arm of an immunoglobulin. Various exemplary assembled hybrid molecules within the scope of the present invention are schematically diagrammed below:

(a) ACH
(b) ACH-ACH
(c) ACH-VHCH-VLCL
(d) ACH-VHCH

wherein each A represents identical or different peptide ligands;
VL is an immunoglobulin light chain variable domain;
VH is an immunoglobulin heavy chain variable domain;
CL is an immunoglobulin light chain constant domain and
CH is an immunoglobulin heavy chain constant domain.

In the interests of brevity, the foregoing structures only show key features; they do not show optional linker domains between the peptide ligand domains and the multimerization domains as described herein below; they do not indicate joining, hinge or other domains of the immunoglobulins, nor are disulfide bonds shown.

Although the presence of an immunoglobulin light chain is not required in the hybrid molecules of the present invention, an immunoglobulin light chain might be present either covalently associated to a peptide ligand-immunoglobulin heavy chain fusion polypeptide, or directly fused to the peptide ligand. In the former case, DNA encoding an immunoglobulin light

chain is typically coexpressed with the DNA encoding the peptide ligand-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs.

The hybrid molecules described herein are most conveniently constructed by fusing the cDNA sequence encoding the peptide ligand portion in-frame to an immunoglobulin cDNA sequence. However, fusion to genomic immunoglobulin fragments can also be used (see, e.g. Aruffo et al., (1990), Cell 61:1303-1313; and Stamenkovic et al. (1991), Cell 66:1133-1144). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequences from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the peptide ligand and the immunoglobulin parts of the hybrid molecule are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells.

Alternatively, and especially in embodiments where the peptide ligand is synthesized by, for example standard solid phase synthesis techniques, the peptide ligand may be linked to the multimerization domain by any of a variety of means familiar to those of skill in the art. Covalent attachment is typically the most convenient, but other forms of attachment may be employed depending upon the application. Examples of suitable forms of covalent attachment include the bonds resulting from the reaction of molecules bearing activated chemical groups with amino acid side chains in the multimerization domain and can be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene).

B. Linker Domains

According to the present invention, the peptide ligand domain is optionally linked to the multimerization domain via an optionally flexible peptide linker. The linker component of the hybrid molecule of the invention does not necessarily participate in but may contribute to the function of the hybrid molecule. Therefore, according to the present invention, the linker domain, is any group of molecules that provides a spatial bridge between the multimerization domain and the peptide ligand domain.

The linker domain can be of variable length and makeup, however, according to the present invention, it is the length of the linker domain and not its structure that is important. The linker domain preferably allows for the peptide ligand domain of the hybrid molecule to bind, substantially free of spacial/ conformational restrictions to the coordinant target molecule. Therefore, the length of the linker domain is dependent upon the character of the two "functional" domains of the hybrid molecule.

One skilled in the art will recognize that various combinations of atoms provide for variable length molecules based upon known distances between various bonds (Morrison, and Boyd, Organic Chemistry, 3rd Ed, Allyn and Bacon, Inc., Boston, MA (1977)). For example, the linker domain may be a polypeptide of variable length. The amino acid composition of the polypeptide determines the character and length of the linker. In a preferred embodiment, the linker molecule comprises a flexible, hydrophilic polypeptide chain. Exemplary, linker domains comprises one or more Gly and or Ser residues, such as those described in the Example sections herein.

C. Bispecific Combinations

According to certain aspects of the invention, bi- or dual-specific compositions comprising at least one peptide ligand domain are envisioned. For example, bispecific antibody compositions have been produced using leucine zippers (Kostelny et al., (1992) J. Immunol., 148(5):1547-1553). The leucine zipper peptides from the Fos and Jun proteins are linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers can be reduced at the hinge region to form monomers and then re-oxidized to form antibody heterodimers. This method can also be utilized for the production of peptide ligand homodimer and heterodimers utilizing the peptide ligands in place of the binding domains of the antibody heterodimers.

Utility

Generally speaking, the hybrid molecules comprising the peptide ligands and their functional derivatives of the present invention can be used in the same applications as, for example, a peptide ligand can be used. The function of the peptide ligand domain generally dictates its use. For example, the peptide ligand may target the hybrid molecule as well as any optional additional functional moiety such as a cytotoxic moiety, to a particular cell type bearing the target molecule. As but one example according to this aspect of the invention, the peptide ligand can bind ErbB2 as described in the Example sections. According to this embodiment the hybrid molecule homes to or targets ErbB2 bearing cell types in vivo. The hybrid molecule may be employed according to this aspect of the invention as a homodimer having a functional Fc domain. The ErbB2 specific hybrid

molecule can further include an additional function moiety such as those described herein below.

As a further example the peptide ligand may bind to and inhibit the activity associated with a particular target molecule. For example a peptide ligand may bind a serine protease such a Factor VII or Factor VIIa involved in the coagulation cascade leading to a demonstrable decrease in the activity associated with Factor VII or Factor FVIIa such as the activation of Factor X.

Of course, some hybrid molecules within the scope of the present invention may be better suited for a particular application than another application. Those skilled in the art will readily ascertain which molecules are appropriate for a given application by using one or more conventional biological assays to determine the biological activity of the peptide ligand or hybrid molecule.

A. Effector Function Engineering

Depending upon its use, it may be desirable to modify the multimerization domain of the hybrid molecules of the present invention with respect to effector function. The skilled artisan can determine appropriate applications in which to employ an effector function associated with an Fc domain or to select or engineer an Fc domain lacking or having diminished effector function.

In a particular example the effectiveness of the hybrid molecule which targets a tumor associated antigen may be enhanced by employing a functional Fc domain in, for example, treating cancer. For example, cysteine residue(s) may be introduced in an immunoglobulin Fc region, thereby allowing interchain disulfide bond formation in this region. The homo- or heterodimeric hybrid molecule thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., (1992) J. Exp Med. 176:1191-1195 and Shopes, B. (1992) J. Immunol. 148:2918-2922. Homodimeric hybrid molecules with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. (1993) Cancer Research 53:2560-2565.

Alternatively, a heterodimeric hybrid molecule can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. (1989) Anti-Cancer Drug Design 3:219-230.

B. Cytotoxic Conjugates

The invention also pertains to conjugates comprising any of the hybrid molecules described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (*e.g.* an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such conjugates have been described. A chemotherapeutic agent is a chemical compound useful in the treatment of cancer including carcinoma, lymphoma, blastoma, sarcoma, and leukemias. Examples of chemotherapeutic agents include, Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside, Cyclophophamide, thiotepa, Busulfan, Cytoxin, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastin, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitosantrone, Vincristine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomydcin, Aminopterin, Dactinomycin, a Mitomycin, Nicotinamide, an Espeeramicin, Melphalan and any related nitrogen mustard, and endocrine therapeutics (such as diethylstilbestrol, Tamoxifen, LHRH-antagonizing drugs, a progestin, an anti-progestin, etc.)

Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes.

A variety of radionuclides are available for the production of radioconjugated peptide ligands or hybrid molecules. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re. Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the hybrid molecules.

Conjugates of the peptide ligand or hybrid molecule and a cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared essentially as described in Vitetta et al. (1987) Science 238: 1098.

In another embodiment, the peptide ligand or hybrid molecules may be conjugated to a "receptor" (such as streptavidin) for utilization in tumor pretargeting wherein the peptide ligand or hybrid molecule-receptor conjugate is administered to a patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (*e.g.* biotin or avidin) which is conjugated to a cytotoxic agent (*e.g.* a radionucleotide).

C. Liposomes

The hybrid molecules or peptide ligands disclosed herein may also be formulated as liposomes. Liposomes containing the hybrid molecules are prepared by methods known in the art, such as described in Epstein et al., (1985) Proc. Natl. Acad. Sci. USA, 82:3688; Hwang et al., (1980) Proc. Natl Acad. Sci. USA, 77:4030; and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. For example, hybrid molecules comprising an immunoglobulin constant domain as described herein can be conjugated to the liposomes as described in Martin *et al. J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon *et al. J. National Cancer Inst.*81(19)1484 (1989).

D. Enzyme Mediated Prodrug Therapy

The peptide ligands or hybrid molecules of the present invention may also be used to target an enzyme moiety linked to the hybrid molecule to a particular cell type bearing the appropriate target molecule by conjugating the peptide ligand or hybrid molecules to a prodrug-activating enzyme which converts a prodrug (*e.g.* a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of the immunoconjugate useful for are those suitable for antibody dependent enzyme mediated prodrug therapy (ADEPT) and includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form. Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for

converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, (1987) Nature 328:457-458). Peptide ligand/hybrid molecule-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

Enzymes can be covalently bound to the hybrid molecules by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the peptide ligand portion of the hybrid molecule of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., (1984) Nature, 312:604-608).

E. Pharmaceutical Compositions

Pharmaceutical compositions which comprise the hybrid molecules of the invention may be administered in any suitable manner, including parental, topical, oral, or local (such as aerosol or transdermal) or any combination thereof. Suitable regimens also include an initial administration by intravenous bolus injection followed by repeated doses at one or more intervals.

Other suitable compositions of the present invention comprise any of the above noted compositions with a pharmaceutically acceptable carrier, the nature of the carrier differing with the mode of administration, for example, in oral administration, usually using a solid carrier and in i.v. administration, a liquid salt solution carrier.

The compositions of the present invention include pharmaceutically acceptable components that are compatible with the subject and the protein of the invention. These generally include suspensions, solutions and elixirs, and most especially biological buffers, such as phosphate buffered saline, saline, Dulbecco's Media, and the like. Aerosols may also be used, or carriers such as starches, sugars, microcrystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents, and the like (in the case of oral solid preparations, such as powders, capsules, and tablets).

As used herein, the term "pharmaceutically acceptable" generally means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans.

The formulation of choice can be accomplished using a variety of the aforementioned buffers, or even excipients including, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin cellulose, magnesium carbonate, and the like. "PEGylation"

of the compositions may be achieved using techniques known to the art (see for example International Patent Publication No. WO92/16555, U.S. Patent No. 5,122,614 to Enzon, and International Patent Publication No. WO92/00748).

A preferred route of administration of the present invention is in the aerosol or inhaled form. The compounds of the present invention, combined with a dispersing agent, or dispersant, can be administered in an aerosol formulation as a dry powder or in a solution or suspension with a diluent.

As used herein, the term "dispersant" refers to a agent that assists aerosolization of the compound or absorption of the protein in lung tissue, or both. Preferably the dispersant is pharmaceutically acceptable. As used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. Suitable dispersing agents are well known in the art, and include but are not limited to surfactants and the like. For example, surfactants that are generally used in the art to reduce surface induced aggregation of the compound, especially the peptide compound, caused by atomization of the solution forming the liquid aerosol may be used. Nonlimiting examples of such surfactants are surfactants such as polyoxyethylene fatty acid esters and alcohols, and polyoxyethylene sorbitan fatty acid esters. Amounts of surfactants used will vary, being generally within the range or 0.001 and 4% by weight of the formulation. In a specific aspect, the surfactant is polyoxyethylene sorbitan monooleate or sorbitan trioleate. Suitable surfactants are well known in the art, and can be selected on the basis of desired properties, depending on the specific formulation, concentration of the compound, diluent (in a liquid formulation) or form of powder (in a dry powder formulation), etc.

Moreover, depending on the choice of the peptide ligand, the desired therapeutic effect, the quality of the lung tissue (e.g., diseased or healthy lungs), and numerous other factors, the liquid or dry formulations can comprise additional components, as discussed further below.

The liquid aerosol formulations generally contain the peptide ligand and a dispersing agent in a physiologically acceptable diluent. The dry powder aerosol formulations of the present invention consist of a finely divided solid form of the peptide ligand and a dispersing agent. With either the liquid or dry powder aerosol formulation, the formulation must be aerosolized. That is, it must be broken down into liquid or solid particles in order to ensure that the aerosolized dose actually reaches the alveoli. In general the mass median dynamic diameter will be 5 micrometers or less in order to ensure that the drug particles reach the lung alveoli (Wearley, L.L., 1991, Crit. Rev. in Ther. Drug Carrier Systems 8:333). The term "aerosol particle" is used herein to describe the liquid or solid particle suitable for pulmonary administration, i.e., that will reach the alveoli.

Other considerations such as construction of the delivery device, additional components in the formulation and particle characteristics are important. These aspects of pulmonary administration of a drug are well known in the art, and manipulation of formulations, aerosolization means and construction of a delivery device require at most routine experimentation by one of ordinary skill in the art.

With regard to construction of the delivery device, any form of aerosolization known in the art, including but not limited to nebulization, atomization or pump aerosolization of a liquid formulation, and aerosolization of a dry powder formulation, can be used in the practice of the invention. A delivery device that is uniquely designed for administration of solid formulations is envisioned. Often, the aerosolization of a liquid or a dry powder formulation will require a propellent. The propellent may be any propellant generally used in the art. Specific nonlimiting examples of such useful propellants are a chloroflourocarbon, a hydrofluorocarbon, a hydochlorofluorocarbon, or a hydrocarbon, including triflouromethane, dichlorodiflouromethane, dichlorotetrafuoroethanol, and 1,1,1,2-tetraflouroethane, or combinations thereof.

In a preferred aspect of the invention, the device for aerosolization is a metered dose inhaler. A metered dose inhaler provides a specific dosage when administered, rather than a variable dose depending on administration. Such a metered dose inhaler can be used with either a liquid or a dry powder aerosol formulation. Metered dose inhalers are well known in the art.

Once the peptide ligand reaches the lung, a number of formulation-dependent factors effect the drug absorption. It will be appreciated that in treating a disease or disorder that requires circulatory levels of the compound, such factors as aerosol particle size, aerosol particle shape, the presence or absence of infection, lung disease or emboli may affect the absorption of the compounds. For each of the formulations described herein, certain lubricators, absorption enhancers, protein stabilizers or suspending agents may be appropriate. The choice of these additional agents will vary depending on the goal. It will be appreciated that in instances where local delivery of the compounds is desired or sought, such variables as absorption enhancement will be less critical.

Liquid Aerosol Formulations

The liquid aerosol formulations of the present invention will typically be used with a nebulizer. The nebulizer can be either compressed air driven or ultrasonic. Any nebulizer known in the art can be used in conjunction with the present invention such as but not limited to: Ultravent, Mallinckrodt, Inc. (St. Louis, MO); the Acorn II nebulizer (Marquest Medical Products, Englewood CO). Other nebulizers useful in

conjunction with the present invention are described in U.S. Patent Nos. 4,624,251 issued November 25, 1986; 3,703,173 issued November 21, 1972; 3,561,444 issued February 9, 1971 and 4,635,627 issued January 13, 1971.

The formulation may include a carrier. The carrier is a macromolecule which is soluble in the circulatory system and which is physiologically acceptable where physiological acceptance means that those of skill in the art would accept injection of said carrier into a patient as part of a therapeutic regime. The carrier preferably is relatively stable in the circulatory system with an acceptable plasma half life for clearance. Such macromolecules include but are not limited to Soya lecithin, oleic acid and sorbetan trioleate, with sorbitan trioleate preferred.

The formulations of the present embodiment may also include other agents useful for protein stabilization or for the regulation of osmotic pressure. Examples of the agents include but are not limited to salts, such as sodium chloride, or potassium chloride, and carbohydrates, such as glucose, golactose or mannose, and the like.

Aerosol Dry Powder Formulations

It is also contemplated that the present pharmaceutical formulation will be used as a dry powder inhaler formulation comprising a finely divided powder form of the peptide ligand and a dispersant. The form of the compound will generally be a lyophilized powder. Lyophilized forms of peptide compounds can be obtained through standard techniques.

In another embodiment, the dry powder formulation will comprise a finely divided dry powder containing one or more compounds of the present invention, a dispersing agent and also a bulking agent. Bulking agents useful in conjunction with the present formulation include such agents as lactose, sorbitol, sucrose, or mannitol, in amounts that facilitate the dispersal of the powder from the device.

Research and Diagnostic Compositions

In a preferred embodiment, the hybrid molecules of the invention are non-covalently adsorbed or covalently bound to a macromolecule, such as a solid support. It will be appreciated that the invention encompasses both macromolecules complexed with the hybrid molecules. In general, the solid support is an inert matrix, such as a polymeric gel, comprising a three dimensional structure, lattice or network of a material. Almost any macromolecule, synthetic or natural, can form a gel in a suitable liquid when suitably cross-linked with a bifunctional reagent. Preferably, the macromolecule selected is convenient for use in affinity chromatography. Most chromatographic matrices used for affinity chromatography are xerogels. Such gels shrink on drying to a compact solid comprising only the gel matrix. When the dried xerogel is resuspended in the liquid, the gel matrix imbibes liquid, swells and returns to the gel state. Xerogels suitable for use herein include polymeric gels, such as cellulose, cross-linked dextrans

(e.g. Sepharose), agarose, cross-linked agarose, polyacrylamide gels, and polyacrylamide-agarose gels.

Alternatively, aerogels can be used for affinity chromatography. These gels do not shrink on drying but merely allow penetration of the surrounding air. When the dry gel is exposed to liquid, the latter displaces the air in the gel. Aerogels suitable for use herein include porous glass and ceramic gels.

Also encompassed herein are the hybrid molecules of the invention coupled to derivatized gels wherein the derivative moieties facilitate the coupling of the hybrid molecules to the gel matrix and avoid stearic hindrance of the peptide ligand-target molecule interaction in affinity chromatography. Alternatively, spacer arms can be interposed between the gel matrix and the hybrid molecules for similar benefits.

Chemical Synthesis

One method of producing the compounds of the invention involves chemical synthesis. This can be accomplished by using methodologies well known in the art (see Kelley, R.F. & Winkler, M.E. in Genetic Engineering Principles and Methods, Setlow, J.K, ed., Plenum Press, N.Y., vol. 12, pp 1-19 (1990), Stewart, J.M. Young, J.D., Solid Phase Peptide Synthesis, Pierce Chemical Co., Rockford, IL (1984); see also U.S. Pat. Nos. 4,105,603; 3,972,859;3,842,067; and 3,862,925).

Peptide ligands of the invention can be conveniently prepared using solid phase peptide synthesis (Merrifield, (1964) J. Am. Chem. Soc., 85:2149; Houghten, (1985) Proc. Natl. Acad. Sci. USA, 82:5132. Solid phase synthesis begins at the carboxy terminus of the putative peptide by coupling a protected amino acid to an inert solid support. The inert solid support can be any macromolecule capable of serving as an anchor for the C-terminus of the initial amino acid. Typically, the macromolecular support is a cross-linked polymeric resin (e.g. a polyamide or polystyrene resin) as shown in Figures 1-1 and 1-2, on pages 2 and 4 of Stewart and Young, supra. In one embodiment, the C-terminal amino acid is coupled to a polystyrene resin to form a benzyl ester. A macromolecular support is selected such that the peptide anchor link is stable under the conditions used to deprotect the α-amino group of the blocked amino acids in peptide synthesis. If a base-labile α-protecting group is used, then it is desirable to use an acid-labile link between the peptide and the solid support. For example, an acid-labile ether resin is effective for base-labile Fmoc-amino acid peptide synthesis as described on page 16 of Stewart and Young, supra. Alternatively, a peptide anchor link and α-protecting group that are differentially labile to acidolysis can be used. For example, an aminomethyl resin such as the phenylacetamidomethyl (Pam) resin works well in conjunction with Boc-amino acid peptide synthesis as described on pages 11-12 of Stewart and Young, supra.

After the initial amino acid is coupled to an inert solid support, the α-amino protecting group of the initial amino acid is removed with, for example, trifluoroacetic acid (TFA) in methylene chloride and neutralizing in, for example, triethylamine (TEA). Following deprotection of the initial amino acid's α-amino group, the next α-amino and sidechain protected amino acid in the synthesis is added. The remaining α-amino and, if necessary, side chain protected amino acids are then coupled sequentially in the desired order by condensation to obtain an intermediate compound connected to the solid support. Alternatively, some amino acids may be coupled to one another to form a fragment of the desired peptide followed by addition of the peptide fragment to the growing solid phase peptide chain.

The condensation reaction between two amino acids, or an amino acid and a peptide, or a peptide and a peptide can be carried out according to the usual condensation methods such as the axide method, mixed acid anhydride method, DCC (N,N'-dicyclohexylcarbodiimide) or DIC (N,N'-diisopropylcarbodiimide) methods, active ester method, p-nitrophenyl ester method, BOP (benzotriazole-1-yl-oxy-tris [dimethylamino] phosphonium hexafluorophosphate) method, N-hydroxysuccinic acid imido ester method, etc, and Woodward reagent K method.

It is common in the chemical synthesis of peptides to protect any reactive side-chain groups of the amino acids with suitable protecting groups. Ultimately, these protecting groups are removed after the desired polypeptide chain has been sequentially assembled. Also common is the protection of the α-amino group on an amino acid or peptide fragment while the C-terminal carboxy group of the amino acid or peptide fragment reacts with the free N-terminal amino group of the growing solid phase polypeptide chain, followed by the selective removal of the α-amino group to permit the addition of the next amino acid or peptide fragment to the solid phase polypeptide chain. Accordingly, it is common in polypeptide synthesis that an intermediate compound is produced which contains each of the amino acid residues located in the desired sequence in the peptide chain wherein individual residues still carry side-chain protecting groups. These protecting groups can be removed substantially at the same time to produce the desired polypeptide product following removal from the solid phase.

α- and ε-amino side chains can be protected with benzyloxycarbonyl (abbreviated Z), isonicotinyloxycarbonyl (iNOC), o-chlorobenzyloxycarbonyl [Z(2Cl)], p-nitrobenzyloxycarbonyl [$Z(NO_2)$], p-methoxybenzyloxycarbonyl [Z(OMe)], t-butoxycarbonyl (Boc), t-amyloxycarbonyl (Aoc), isobornyloxycarbonyl, adamantyloxycarbonyl, 2-(4-biphenyl)-2-propyloxycarbonyl (Bpoc), 9-fluorenylmethoxycarbonyl (Fmoc), methylsulfonyethoxycarbonyl (Msc), trifluoroacetyl, phthalyl, formyl, 2-nitrophenylsulphenyl (NPS), diphenylphosphinothioyl (Ppt), and dimethylphosphinothioyl (Mpt) groups, and the like.

Protective groups for the carboxy functional group are exemplified by benzyl ester (OBzl), cyclohexyl ester (Chx), 4-nitrobenzyl ester (ONb), t-butyl ester (Obut), 4-pyridylmethyl ester (OPic), and the like. It is often desirable that specific amino acids such as arginine, cysteine, and serine possessing a functional group other than amino and carboxyl groups are protected by a suitable protective group. For example, the guanidinc group of arginine may be protected with nitro, p-toluenesulfonyl, benzyloxycarbonyl, adamantyloxycarbonyl, p-methoxybenzesulfonyl, 4-methoxy-2,6-dimethylbenzenesulfonyl (Nds), 1,3,5-trimethylphenysulfonyl (Mts), and the like. The thiol group of cysteine can be protected with p-methoxybenzyl, trityl, and the like.

Many of the blocked amino acids described above can be obtained from commercial sources such as Novabiochem (San Diego, CA), Bachem CA (Torrence, CA) or Peninsula Labs (Belmont, CA).

Stewart and Young, supra, provides detailed information regarding procedures for preparing peptides. Protection of α-amino groups is described on pages 14-18, and side chain blockage is described on pages 18-28. A table of protecting groups for amine, hydroxyl and sulfhydryl functions is provided on pages 149-151.

After the desired amino acid sequence has been completed, the peptide can be cleaved away from the solid support, recovered and purified. The peptide is removed from the solid support by a reagent capable of disrupting the peptide-solid phase link, and optionally deprotects blocked side chain functional groups on the peptide. In one embodiment, the peptide is cleaved away from the solid phase by acidolysis with liquid hydrofluoric acid (HF), which also removes any remaining side chain protective groups. Preferably, in order to avoid alkylation of residues in the peptide (for example, alkylation of methionine, cysteine, and tyrosine residues), the acidolysis reaction mixture contains thio-cresol and cresol scavengers. Following HF cleavage, the resin is washed with ether, and the free peptide is extracted from the solid phase with sequential washes of acetic acid solutions. The combined washes are lyophilized, and the peptide is purified.

Disulfide Linked Peptides

As described above, some embodiments of the invention include cyclized peptide ligands. Peptide ligands may be cyclized by formation of a disulfide bond between cysteine residues. Such peptides can be made by chemical synthesis as described above and then cyclized by any convenient method used in the formation of disulfide linkages. For example, peptides can be recovered from solid phase synthesis with sulfhydryls in reduced form, dissolved in a dilute solution wherein the intramolecular cysteine concentration exceeds the intermolecular cysteine concentration in order to optimize intramolecular disulfide bond formation, such as a peptide concentration of 25 mM to 1 μM, and preferably 500 μM to 1 μM, and more

preferably 25 μM to 1 μM, and then oxidized by exposing the free sulfhydryl groups to a mild oxidizing agent that is sufficient to generate intramolecular disulfide bonds, e.g. molecular oxygen with or without catalysts such as metal cations, potassium ferricyanide, sodium tetrathionate, etc. In one embodiment, the peptides are cyclized as described in Example 2 below. Alternatively, the peptides can be cyclized as described in Pelton et al., (1986) J. Med. Chem., 29:2370-2375.

Cyclization can be achieved by the formation for example of a disulfide bond or a lactam bond between first Cys and a second Cys. Residues capable of forming a disulfide bond include for example Cys, Pen, Mpr, and Mpp and its 2-amino group-containing equivalents. Residues capable of forming a lactam bridge include for example, asp Glu, Lys, Orn, αβ-diaminobutyric acid, diaminoacetic acid, aminobenzoic acid and mercaptobenzoic acid. The compounds herein can be cyclized for example via a lactam bond which can utilize the side chain group of a non-adjacent residue to form a covalent attachment to the N-terminus amino group of Cys1 or other amino acid. Alternative bridge structures also can be used to cyclize the compounds of the invention, including for example, peptides and peptidomimetics, which can cyclize via S-S, CH2-S, CH2-O-CH2, lactam ester or other linkages.

Recombinant Synthesis

In a further embodiment, the present invention encompasses a composition of matter comprising isolated nucleic acid, preferably DNA, encoding a peptide described herein. DNAs encoding the peptides of the invention can be prepared by a variety of methods known in the art. These methods include, but are not limited to, chemical synthesis by any of the methods described in Engels et al., (1989) Agnew. Chem. Int. Ed. Engl., 28:716-734, the entire disclosure of which is incorporated herein by reference, such as the triester, phosphite, phosphoramidite and H-phosphonate methods. In one embodiment, codons preferred by the expression host cell are used in the design of the encoding DNA. Alternatively, DNA encoding the peptide can be altered to encode one or more variants by using recombinant DNA techniques, such as site specific mutagenesis (Kunkel et al., (1991) Methods Enzymol. 204:125-139; Carter, P., et al., (186) Nucl. Acids. Res. 13:4331; Zoller, M. J. et al., (1982) Nucl. Acids Res. 10:6487), cassette mutagenesis (Wells, J. A., et al., (1985) Gene 34:315), restriction selection mutagenesis (Wells, J. A., et al., (1986) Philos. Trans, R. Soc. London SerA 317, 415), and the like.

The invention further comprises an expression control sequence operably linked to the DNA molecule encoding a peptide of the invention, and an expression vector, such as a plasmid, comprising the DNA molecule, wherein the control sequence is recognized by a host cell transformed with the vector. In general, plasmid vectors contain replication and control

sequences which are derived from species compatible with the host cell. The vector ordinarily carries a replication site, as well as sequences which encode proteins that are capable of providing phenotypic selection in transformed cells.

For expression in prokaryotic hosts, suitable vectors include pBR322 (ATCC No. 37,017), phGH107 (ATCC No. 40,011), pBO475, pS0132, pRIT5, any vector in the pRIT20 or pRIT30 series (Nilsson and Abrahmsen, (1990) Meth. Enzymol., 185:144-161), pRIT2T, pKK233-2, pDR540 and pPL-lambda. Prokaryotic host cells containing the expression vectors of the present invention include *E. coli* K12 strain 294 (ATCC NO. 31446), *E coli* strain JM101 (Messing et al.,(1981) Nucl. Acid Res., 9:309), *E. coli* strain B, *E. coli* strain χ1776 (ATCC No. 31537), *E. coli* c600 (Appleyard, *Genetics*, **39**: 440 (1954)), *E. coli* W3110 (F-, gamma-, prototrophic, ATCC No. 27325), *E. coli* strain 27C7 (W3110, *tonA, phoA E15, (argF-lac)169, ptr3, degP41, ompT, kan*^{r}) (U.S. Patent No. 5,288,931, ATCC No. 55,244), *Bacillus subtilis*, *Salmonella typhimurium*, *Serratia marcesans*, and *Pseudomonas* species.

In addition to prokaryotes, eukaryotic organisms, such as yeasts, or cells derived from multicellular organisms can be used as host cells. For expression in yeast host cells, such as common baker's yeast or *Saccharomyces cerevisiae*, suitable vectors include episomally replicating vectors based on the 2-micron plasmid, integration vectors, and yeast artificial chromosome (YAC) vectors. For expression in insect host cells, such as Sf9 cells, suitable vectors include baculoviral vectors. For expression in plant host cells, particularly dicotyledonous plant hosts, such as tobacco, suitable expression vectors include vectors derived from the Ti plasmid of *Agrobacterium tumefaciens*.

However, interest has been greatest in vertebrate host cells. Examples of useful mammalian host cells include monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., (1977) J. Gen Virol., 36:59); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, (1980) Proc. Natl. Acad. Sci. USA, 77:4216); mouse sertoli cells (TM4, Mather, (1980) Biol. Reprod., 23:243-251); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., (1982) Annals N.Y. Acad. Sci., 383:44-68); MRC 5 cells; FS4 cells; and a human hepatoma cell line (Hep G2). For expression in mammalian host cells, useful vectors include vectors derived from SV40, vectors derived from cytomegalovirus such as the pRK vectors, including pRK5 and pRK7 (Suva et al., (1987) Science, 237:893-896;

EP 307,247 (3/15/89), EP 278,776 (8/17/88)) vectors derived from vaccinia viruses or other pox viruses, and retroviral vectors such as vectors derived from Moloney's murine leukemia virus (MoMLV).

Optionally, the DNA encoding the peptide of interest is operably linked to a secretory leader sequence resulting in secretion of the expression product by the host cell into the culture medium. Examples of secretory leader sequences include stII, ecotin, lamB, herpes GD, lpp, alkaline phosphatase, invertase, and alpha factor. Also suitable for use herein is the 36 amino acid leader sequence of protein A (Abrahmsen et al., (1985) EMBO J., 4:3901).

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ precipitation and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook et al., Molecular Cloning (2nd ed.), Cold Spring Harbor Laboratory, NY (1989), is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., (1983) Gene, 23:315 and WO 89/05859 published 29 June 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method described in sections 16.30-16.37 of Sambrook et al., supra, is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen et al., (1977) J. Bact., 130:946 and Hsiao et al., (1979) Proc. Natl. Acad. Sci. (USA), 76:3829. However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or by protoplast fusion may also be used.

Prokaryotic host cells used to produce the present peptides can be cultured as described generally in Sambrook et al., supra.

The mammalian host cells used to produce peptides of the invention can be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, (1979) Meth. in Enz., 58:44, Barnes and Sato, (1980) Anal. Biochem., 102:255, U.S. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. Re. 30,985; or U.S. 5,122,469, the disclosures of all of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The host cells referred to in this disclosure encompass cells in in vitro culture as well as cells that are within a host animal.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all citations in the specification are expressly incorporated herein by reference.

EXAMPLES

EXAMPLE I

Peptide-Fc Fusions

Methods

Construction of TF151-Fc and HER2-Fc (1.1FI-Fc) expression vectors—Standard recombinant DNA techniques were used for the construction of recombinant transfer vectors based on the vector pVL1393 (Pharmigen) (Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, second Ed., Cold Spring Harbor Laboratory Press, New York; O'Reilly, D. R., Miller, L. K., and Luckow, V. A. (1994) *Baculovirus Expression Vectors: A Laboratory Manual*, Oxford University Press, New York). The pVL1393 derived plasmid pbPH.His was linearized with *Nco* I and *Sma* I and treated with shrimp alkaline phosphatase (Dwyer, M. A. et al. (1999) J. Biol. Chem. 274:9738-9743). The Fc portion of the human IgG1 was obtained as a 700 base pair fragment by restriction digestion using *Nde* I and subsequent treatment with Klenow and *Nco* I of another pVL1393 derived plasmid pVL1393.IgG. The signal sequence for MIP.5 was introduced before

the Fc sequence as a PCR fragment digested with *EcoR* I, included within the fragment is an *Asc* I site. The *Asc* I site occurs following the putative signal sequence cleavage site. Following ligation, competent *E. coli* XL-1 Blue were transformed and bacteria were selected for the correct recombinant plasmid (pVL1393.MIP.5sig.Fc) by DNA sequence analysis. Then, pVL1393.MIP.5sig.Fc was linearized with *Asc* I and *Stu* I and treated with shrimp alkaline phosphatase. The linearized vector was then ligated with a synthetic piece of DNA with compatible ends. The synthetic DNA inserts were formed by annealing 2 oligos with the sequences: 5'-GCC GGA GCT CCC GCC TCC GCC CTC CAC GAA CTG GCA GTA CCA CCT GTC GAT TCT GGG GTT GTC GCA CAG GGC GCC CAC GG-3' (SEQ ID NO:11) and 5'-CGC GCC GTG GGC GCC CTG TGC GAC AAC CCC AGA ATC GAC AGG TGG TAC TGC CAG TTC GTG GAG GGC GGA GGC GGG AGC TCC GGC-3' (SEQ ID NO:12) coding for peptide sequence TF151 (Table 1) including a GGGSSG (SEQ ID NO:13) linker or by annealing 2 oligos with the sequences: 5'-CGC GCC CAG GTG TAC GAG TCC TGG GGA TGC ATC GGC CCC GGC TGC GCC TGC CTG CAG GCC TGC CTG GGA GGC GGG AGC TCC GGC-3' (SEQ ID NO:14) and 5'-GCC GGA GCT CCC GCC TCC CAG GCA GGC CTG CAG GCA GGC GCA GCC GGG GCC GAT GCA TCC CCA GGA CTC GTA CAC CTG GG-3' (SEQ ID NO:15) coding for peptide sequence 1.1FI (Table 1) including a GGGSSG (SEQ ID NO:13) linker.

Following ligation, competent *E. coli* XL-1 Blue were transformed and bacteria were selected for the correct recombinant plasmid (termed pVL.1393.MIP5.TF151-Fc or pVL.1393.MIP5.1.1FI-Fc) by DNA sequence analysis using the dRhodamine dye-terminator method and an Applied Biosystems ABI Model 373 automated DNA sequencer. Recombinant transfer vector was purified using a Qiagen Mini-Prep and used for construction of recombinant baculovirus.

Recombinant baculovirus, AcNpV.TF151-Fc, was generated following cotransfection of Sf9 cells with the transfer vector and the linearized wild type baculovirus DNA (*Autographa californica* nuclear polyhedrosis virus (AcNpV), Pharmingen). A primary amplification of the recombinant baculovirus, AcNpV.TF151-Fc, achieved detectable protein expression. Subsequent plaque-purification and titering of the viral stock was performed by plaque assays. Standard methods were utilized as previously described (O'Reilly, D. R., Miller, L. K., and Luckow, V. A. (1994) *Baculovirus Expression Vectors: A Laboratory Manual*, Oxford University Press, New York).

Cell Culture—Adherent cultures of *Spodeptera frugiperda* (Sf9) insect cells (ATCC CRL 1711) were maintained at 28 °C in Hink's TNM-FH insect medium supplemented GRACE's (JRH Biosciences, #51942-78P), with glutamine, streptomycin/penicillin, and 10% fetal bovine serum (heat inactivated for 30 min at 56 °C). Cultures were passaged every 3 days. Spinner cultures of High Five™ cells (Trichoplusia ni, BT1.TN.SB1-4 (Invitrogen)) (500 ml at 2.0 x 10^6 cells/ml) were infected at a multiplicity of infection of 0.5 and harvested 60 h posttransfection. Suspension cultures were maintained in

spinner flasks at 28 °C using ESF-921 protein free insect cell culture medium (Expression Systems LLC, #96-001). Cultures were passaged every 3 days to a starting cell density of 10^6 cells/ml.

Peptide-Fc Purification—Following the optimized infection protocol, the High Five™ cells were removed by centrifugation at 800 x g at 4 °C for 10 min. The clarified supernatant (0.5 L) was filtered using a 0.45 µ Nalgene filter and applied to a 0.5 ml Hi-Trap Protein A Column (Amersham Pharmacia Biotech) equilibrated with PBS (phosphate buffered saline) at 25 °C. After washing with 20 ml of PBS, the column was eluted with 3 ml of 0.2 N HOAc and fractions containing peptide-Fc were lyophilized and stored at 4 °C.

SDS-PAGE —Samples were analyzed reduced and unreduced on a 4-20% Tris-glycine SDS-PAGE (Novex) along with prestained protein molecular weight markers (SeaBlue, Novex) using the method of Laemmli (Laemmli, U. K. (1970) Nature 227:680-685).

Protein Sequencing—TF151-Fc and 1.1FI-Fc purified from the infected Sf9 cell supernatants were subjected to SDS-PAGE, and then transferred to a PVDF membrane. Electroblotting onto Millipore Immobilon-PSQ membranes was carried out for 1 h at 250 mA constant current in a BioRad Trans-Blot transfer cell (Matsudaira, P. (1987) J. Biol. Chem. 262: 10035-10038). The PVDF membrane was stained with 0.1% Coomassie Blue R-250 in 50% methanol, 0.5 min and destained for 2-3 min with 10% acetic acid in 50% methanol. The membrane was thoroughly washed with water and allowed to dry before storage at -20 °C. The TF151-Fc and 1.1FI-Fc bands at about 50 kD were each cut out and the first 11 residues were sequenced using a model 494A Applied Biosystems sequencer equipped with an on-line PTH analyzer. Peaks were integrated with Justice Innovation software using Nelson Analytical 760 interfaces. Sequence interpretation was performed on a DEC alpha (Henzel, W. J., Rodriguez, H., and Watanabe, C. (1987) J. Chromatog. 404: 41-52).

Assays for TF151-Fc

FVIIa Binding ELISA—The ability of the TF151-Fc fusion peptides to compete with TF147b,a biotinylated version of TF76, having the amino acid sequence Ac-ALCDDPRVDRWYCQFVEGSK-acaBi amide (SEQ ID NO:10) (Table 1) known to bind FVIIa at the same site as TF151 for binding to FVIIa was monitored using a FVIIa Binding ELISA. Microtiter plates were coated overnight with 2 µg/ml recombinant human FVIIa in 50 mM ammonium bicarbonate pH 9 at 4 °C; all other steps were performed at room temperature. Plates were then blocked with 1 % BSA in Assay Buffer (50 mM HEPES, pH 7.2, 5 mM $CaCl_2$, 150 mM NaCl). Dilutions of TF151-Fc in Assay Buffer plus 0.05 % Tween 20 were added to the microtiter plate along with 20 nM TF147b for 1 h. The microtiter plate was washed 3 times with 300 µl Assay Buffer plus 0.05 % Tween 20 and the TF147b bound was detected with a Strepavidin/HRP conjugate (Streptavidin-POD, Roche Molecular Biochemicals). The amount of HRP bound

was measured using ABTS/H_2O_2 substrate (Kirkegaard and Perry Laboratories) and monitoring the absorbance at 405 nm. The absorbance at 405 nm was plotted versus the concentration of peptide originally added to the well. Sigmoidal curves were fit to a four parameter equation by nonlinear regression analysis (Marquardt, J. Soc. Indust. Appl. Math. 11:431-441 (1963); the concentration of TF151-Fc required to give a half-maximal signal in the assay was calculated from the curves and is referred to as the IC_{50} value.

FX Activation Assay– Activation of FX by TF-FVIIa was monitored at room temperature as a function of TF151-Fc concentration. Each assay sample contained 100 µl of 460 pM relipidated TF_{1-243} (TF_{PC}) (Kelley, R. F. et al. (1997) Blood 89:3219-3227) and 30 pM FVIIa in HBS/Ca buffer (20 mM HEPES, pH 7.4, 5 mM $CaCl_2$, 150 mM NaCl, 0.1 % PEG 8000); after 20 min, 25 µl of peptide diluted in HBS/Ca Buffer was added. Following a 30 min incubation the reaction was initiated by the addition of 25 µl of 1 µM FX in HBS/Ca (Note: this yields a final concentration of 306 pM TF_{PC}, 20 pM FVIIa, and 166 nM FX). For kinetic analysis, the final concentration of FX was varied from between 20 and 500 nM. Aliquots of 25 µl were removed at 1, 3, 5, 7 and 9 min and quenched in 25 µl of 50 mM EDTA. The FXa generated in each aliquot could be measured by the addition of 100 µl of 250 nM Spectrozyme fXa (American Diagnostica), 50 mM Tris, pH 8, 50 mM NaCl, 0.0025 % Triton X-100. The rate of FXa generated at each concentration of TF151-Fc was proportional to the initial slope of the absorbance at 405 nm vs. time. Sigmoidal curves were fit to a four parameter equation by nonlinear regression analysis (Marquardt, J. Soc. Indust. Appl. Math. 11:431-441 (1963); the concentration of each peptide required to give a half-maximal signal in the assay was calculated from the curves and is referred to as the IC_{50} value.

Clotting Assays - The prothrombin time (PT) clotting time assay was performed in citrated pooled normal plasmas (human or various animal species). Clotting times were determined using an ACL 300 Automated Coagulation Analyzer (Coulter Corp., Miami, FL) and commercially available reagents as follows.

For the PT assay, aqueous solutions of (TF151-Fc) at various concentrations are added to citrated pooled normal plasma in a ratio of 1 part inhibitor to 9 parts plasma. Following a 30 min incubation, these mixtures were added to the sample cups of a ACL 300 Analyzer. Innovin® (Dade International Inc., Miami, FL), a mixture of human relipidated tissue factor and Ca^{2+} ions was added to the reagent cup. Precise volumes of sample and Innovin® (50 µl sample, 100 µl Innovin) were automatically transferred to cells of an acrylic rotor pre-equilibrated to 37 °C. Following a 2 min incubation period, coagulation was initiated by mixing the two components together by centrifugation. Coagulation was monitored optically and clotting time was reported in seconds. In this system, the clotting time of

control plasmas (plasma plus inhibitor diluent) was typically 8 to 10 seconds. The fold prolongation was the clotting time of the inhibitor relative to the clotting time of the control.

Assays for 1.1FI-Fc (HER2-Fc)

HER2 Phage Binding Assay- Inhibition of binding of phage displaying a single copy of peptide 1.1FI (Table 1) on their surface to the immobilized extracellular domain of Erb2 (HER2-ECD, Hudziak et al. (1991) J. Biol. Chem. 266:24109-15) was monitored using a phage ELISA. HER2-ECD was immobilized directly to Maxisorp plates (Nunc) in 50 mM ammonium bicarbonate, pH 9.3, using 5 µg/ml, overnight at 4 °C. Wells were blocked using PBS containing 1% BSA (PBS-BSA) for 1 h at 25 °C. Dilutions of 1.1FI-Fc in PBS-BSA were tested for their ability to block the binding of 1.1FI displaying phage to the immobilized HER2-ECD. The microtiter plate was washed with PBS containing 0.05 % Tween20 (PBS-Tween) and the phage bound to HER2-ECD were detected with an anti-gVIII/HRP monoclonal antibody conjugate (Amersham Pharmacia Biotech). The amount of HRP bound was measured using ABTS/H_2O_2 substrate and monitoring the change at 405 nm.

HER2 Competition ELISA- 1.1FI-Fc binding to HER2-ECD was monitored using a competition ELISA. Samples were titered in PBS-BSA and tested for their ability to block the binding of 40 nM biotinylated 1.1.FI-Z (Table 1) to HER2-ECD immobilized on microtiter plates as described above. Following a 1 h incubation the plate was washed with PBS-Tween and Streptavidin/HRP conjugate (Streptavidin-POD, Roche Molecular Biochemicals) was added for 30 min. Plates were washed again with PBS-Tween and the bound HRP was assayed using ABTS/H_2O_2 substrate (Kirkegaard & Perry Laboratories) and the absorbance at 405 nm was monitored. The absorbance at 405 nm was plotted versus the concentration of 1.1FI-Fc originally added to the well. Sigmoidal curves were fit to a four parameter equation by nonlinear regression analysis (Marquardt, J. Soc. Indust. Appl. Math. 11:431-441 (1963); the concentration of 1.1FI-Fc required to give a half-maximal signal in the assay was calculated from the curves and is referred to as the IC_{50} value.

Table 1

HER2 peptide ligands

1.1FI **QVYESWGCIGPGCACLQAL** (SEQ ID NO:16)

1.1FI-phage **QVYESWGCIGPGCACLQACL**-GGGSGGGASGGGSGSG-DFDYEK...(gIII protein) (SEQ ID NO:17)

1.1FI-Fc (HER2-Fc) SQAQRRA- **QVYESWGCIGPGCACLQACL**-GGGSSG-PDKTHTCPPCPA...(Fc,IgG isotype 1) (SEQ ID NO:18)

1.1FI-Z **QVYESWGCIGPGCACLQACL**-GGGRGG-AQHD...(Z) (SEQ ID NO:19)

1.1FI-Z-biotin as above, but randomly biotinylated through the Z domain

TF peptide ligands

TF151 **Ac-ALCDNPRIDRWYCQFVEG-NH2** (SEQ ID NO:20)

TF147b **Ac-ALCDDPRVDRWYCQFVEGSK*-acaBi-amide** (SEQ ID NO:10)

TF76 **Ac-ALCDDPRVDRWYCQFVEG-amide** (SEQ ID NO:21)

TF151-Fc SQAQRRA-**VGALCDNPRIDRWYCQFVEG**-GGGSSG-PDKTHTCPPCPA...(Fc,IgG isotype 1) (SEQ ID NO:22)

Ac denotes CH_3CO-modified N-terminus; NH_2 denotes NH_2 modified C-terminus; aca denotes aminocaproic acid; Bi denotes biotin; * denotes that the ε amino group of lysine was derivatized.

RESULTS

Protein Characterization of Peptide ligand-Fc fusions- Following purification, approximately 8 mg of control-Fc (an Fc lacking a fused peptide ligand), TF151-Fc or 1.1FI-Fc was obtained from a 500 ml culture. SDS-PAGE analysis of the reduced and unreduced fusions revealed bands at about 30 and about 60 kDa respectively, suggesting the association of two peptide-Fc monomers to form a dimer (**Figure 4**). N-terminal sequence analysis of TF151-Fc or 1.1FI-Fc revealed the following sequences: SQAQRRAVGAL.... (TF151-Fc) (SEQ ID NO:23) and SQAQRRAQVYE... (1.1FI-Fc)(SEQ ID NO:24) indicating expression of the peptides as fusions to the Fc.

Functional Characterization of Peptide Ligand-Fc Fusions-

TF151-Fc Activity- The ability of TF151-Fc to compete with TF147b a biotinylated version of TF76, for binding to FVIIa was monitored using a FVIIa Binding ELISA. The inhibition of TF147b binding to FVIIa was comparable to peptide TF151 (Table 1) and is shown in **Figure 5**; TF151 and the TF151-Fc fusion had IC_{50} values of 2 and 3 nM, respectively. The TF151-

Fc fusion was also an effective inhibitor of FX activation by TF-FVIIa in a FX activation assay (**Figure 6**); control-Fc and 1.1FI-Fc had no effect in this assay. The ability to block FX activation with TF151-Fc was also reflected by its ability to prolong the prothrombin time in human plasma (**Figure 7**). The potency of TF151-Fc was comparable to peptide TF151 in this assay.

Characterization of 1.1FI-Fc Fusion- Using the HER2 Phage Binding assay, the 1.1FI-Fc was found to block 1.1FI phage binding to immobilized HER2-ECD, with an IC_{50} value of 3 nM which is similar to 1.1.FI-Z (Table 1) as shown in Figure 8. When tested in the HER2 Competition ELISA, 1.1FI-Fc also had a similar IC_{50} value to 1.1FI-Z (**Figure 9**).

WHAT IS CLAIMED IS:

1. A polypeptide which comprises:
   (a) a peptide ligand and
   (b) an immunoglobulin constant region multimerization domain.

2. The polypeptide of claim 1 wherein the peptide ligand is a non-naturally occurring amino acid sequence of less than 50 amino acid residues.

3. The polypeptide of claim 2 wherein the peptide ligand is between about 10 and about 30 amino acid residues.

4. The polypeptide of claim 3 wherein the immunoglobulin constant region multimerization domain comprises the CH3 domain of an IgG.

5. The polypeptide amino acid sequence of claim 4 wherein the peptide ligand is fused C-terminally to the N-terminus of the immunoglobulin constant region multimerization domain.

6. The polypeptide amino acid sequence of claim 5 wherein the immunoglobulin constant region multimerization domain is obtained from IgG1, IgG2, IgG3, IgG4.

7. The polypeptide amino acid sequence of claim 6 wherein the immunoglobulin constant region multimerization domain is the constant domain of an IgG1 heavy chain.

8. A dimer comprising the polypeptide amino of claim 7 and a second immunoglobulin constant region multimerization domain.

9. The dimer of claim 8 wherein the immunoglobulin constant region multimerization domains combine to form a functional Fc domain.

10. The dimer of claim 9 selected from the group consisting of
   (a) ACH-ACH;
   (b) ACH-VHCH-VLCL and
   (c) ACH-VHCH,
wherein A represents identical or different peptide ligands;
VL is an immunoglobulin light chain variable domain;
VH is an immunoglobulin heavy chain variable domain;
CL is an immunoglobulin light chain constant domain and
CH is an immunoglobulin heavy chain constant domain.

11. Nucleic acid encoding a polypeptide amino acid sequence according to

any one of claims 1 to 7.

12. A method for preparing a polypeptide according to claim 1 comprising recovering the polypeptide from a culture of a host cell transfected with the nucleic acid of claim 11.

13. The method of claim 12 wherein the polypeptide is recovered by adsorption onto an affinity matrix.

14. A replicable vector comprising the nucleic acid of claim 11.

15. A host cell comprising the vector of claim 14.

16. The host cell of claim 15 which further comprising nucleic acid a second immunoglobulin heavy chain.

17. A composition comprising the polypeptide of claim 1 and a cytotoxic agent.

18. The composition of claim 17 wherein the cytotoxic agent is covalently linked to the polypeptide.

19. The composition of claim 18 wherein the cytotoxic agent is a chemotherapeutic agent.

20. The composition of claim 19 wherein the cytotoxic agent is a toxin.

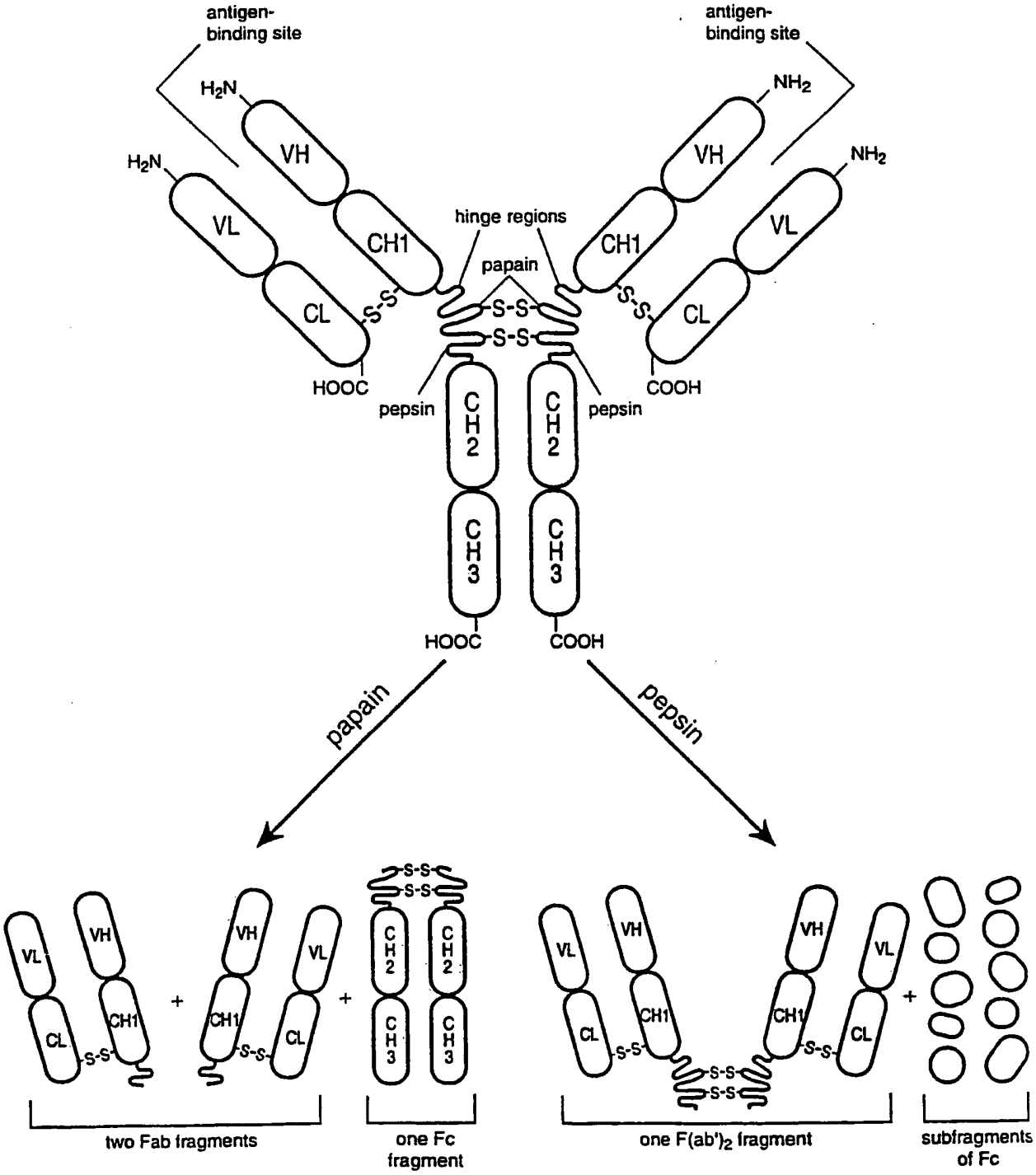
21. The composition of claim 20 wherein the cytotoxic agent is a radioactive isotope.

22. A composition comprising the polypeptide of claim 1 and an enzyme component.

23. The composition of claim 22 wherein the enzyme component is conjugated to the polypeptide.

24. The composition of claim 23 wherein the enzyme component is a pro-drug activating enzyme.

25. The composition of any of claims 17 to 24 further comprising a pharmaceutically acceptable excipient.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antigen-binding site
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VH
VL
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papain
S-S
HOOC
COOH
pepsin
CH2
CH3
papain
pepsin
two Fab fragments
one Fc fragment
one F(ab')2 fragment
subfragments of Fc

Fig.1

```
          230        240        250        260        270
humIgG1     PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
humIgG2     PAP-PVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYV
humIgG3     PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFKWYV
humIgG4     PAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYV
murIgG1     ---TVPEVSSVFIFPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFV
murIgG2A    PAPNLLGGPSVFIFPPKIKDVLMISLSPIVTCVVVDVSEDDPDVQISWFV
murIgG2B    PAPNLEGGPSVFIFPPNIKDVLMISLTPKVTCVVVDVSEDDPDVQISWFV
murIgG3     PPGNILGGPSVFIFPPKPKDALMISLTPKVTCVVVDVSEDDPDVHVSWFV

          280        290        300        310        320
humIgG1     DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
humIgG2     DGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLP
humIgG3     DGVEVHNAKTKPREEQFNSTFRVVSVLTVLHQDWLNGKEYKCKVSNKALP
humIgG4     DGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLP
murIgG1     DDVEVHTAQTQPREEQFNSTFRSVSELPIMHQDCLNGKEFKCRVNSAAFP
murIgG2A    NNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNKDLP
murIgG2B    NNVEVHTAQTQTHREDYNSTIRVVSHLPIQHQDWMSGKEFKCKVNNKDLP
murIgG3     DNKEVHTAWTQPREAQYNSTFRVVSALPIQHQDWMRGKEFKCKVNNKALP

          330        340        350        360        370
humIgG1     APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAV
                                              D L
humIgG2     APIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAV
humIgG3     APIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAV
humIgG4     SSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAV
murIgG1     APIEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFPEDITV
murIgG2A    APIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYV
murIgG2B    SPIERTISKPKGLVRAPQVYTLPPPAEQLSRKDVSLTCLVVGFNPGDISV
murIgG3     APIERTISKPKGRAQTPQVYTIPPPREQMSKKKVSLTCLVTNFFSEAISV

          380        390        400        410        420
humIgG1     EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH
humIgG2     EWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH
humIgG3     EWESSGQPENNYNTTPPMLDSDGSFFLYSKLTVDKSRWQQGNIFSCSVMH
humIgG4     EWZSNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMH
murIgG1     EWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNWEAGNTFTCSVLH
murIgG2A    EWTNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVH
murIgG2B    EWTSNGHTEENYKDTAPVLDSDGSYFIYSKLNMKTSKWEKTDSFSCNVRH
murIgG3     EWERNGELEQDYKNTPPILDSDGTYFLYSKLTVDTDSWLQGEIFTCSVVH

          430        440
humIgG1     EALHNHYTQKSLSLSPGK
humIgG2     EALHNHYTQKSLSLSPGK
humIgG3     EALHNRFTQKSLSLSPGK
humIgG4     EALHNHYTQKSLSLSLGK
murIgG1     EGLHNHHTEKSLSHSPGK
murIgG2A    EGLHNHHTTKSFSRTPGK
murIgG2B    EGLKNYYLKKTISRSPGK
murIgG3     EALHNHHTQKNLSRSPGK
```

Fig. 2A

Percent Identity Among Fc Sequences

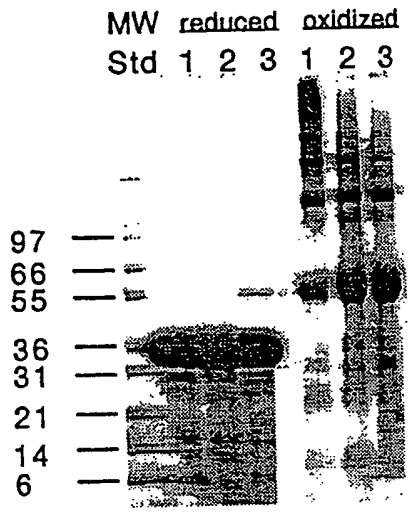
	1	2	3	4	5	6	7	8
1. humIgG1	-	94	94	94	64	66	63	68
2. humIgG2		-	93	92	65	63	60	67
3. humIgG3			-	91	64	64	61	67
4. humIgG4				-	62	64	61	64
5. murIgG1					-	65	61	67
6. murIgG2A						-	77	70
7. murIgG2B							-	68
8. murIgG3								-

Fig. 2B

```
          230        240        250        260        270
humIgG1     PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
humIgG2     PAP-PVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYV
humIgG3     PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFKWYV
humIgG4     PAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYV
               ****                                  *     * *

          280        290        300        310        320
humIgG1     DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
humIgG2     DGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLP
humIgG3     DGVEVHNAKTKPREEQFNSTFRVVSVLTVLHQDWLNGKEYKCKVSNKALP
humIgG4     DGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLP
                           *    *         *                   *

          330        340        350        360        370
humIgG1     APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAV
                                                D L
humIgG2     APIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAV
humIgG3     APIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAV
humIgG4     SSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAV
            **       *                        *

          380        390        400        410        420
humIgG1     EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH
humIgG2     EWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH
humIgG3     EWESSGQPENNYNTTPPMLDSDGSFFLYSKLTVDKSRWQQGNIFSCSVMH
humIgG4     EWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMH
               *        *    *                  *          *  *

          430        440
humIgG1     EALHNHYTQKSLSLSPGK
humIgG2     EALHNHYTQKSLSLSPGK
humIgG3     EALHNRFTQKSLSLSPGK
humIgG4     EALHNHYTQKSLSLSLGK
               **          *
```

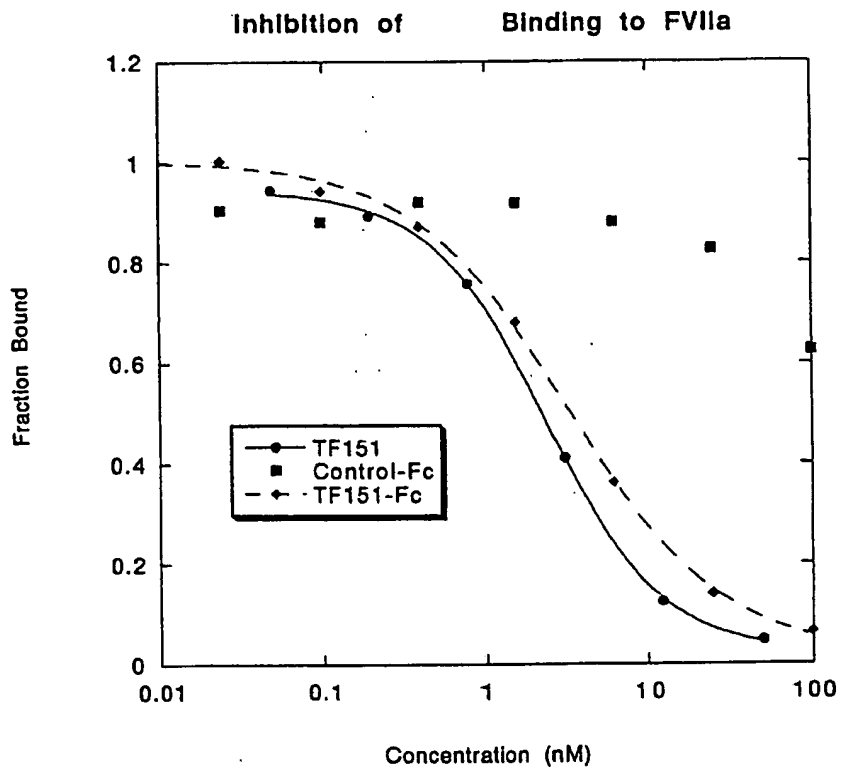
Fig. 3

Figure 4



1 = Control Fc (no peptide fusion)
2 = HER2-Fc fusion
3 = TF151-Fc fusion

TF1476


Inhibition of Binding to FVIIa
Fraction Bound
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1
0.8
0.6
0.4
0.2
0
0.01
0.1
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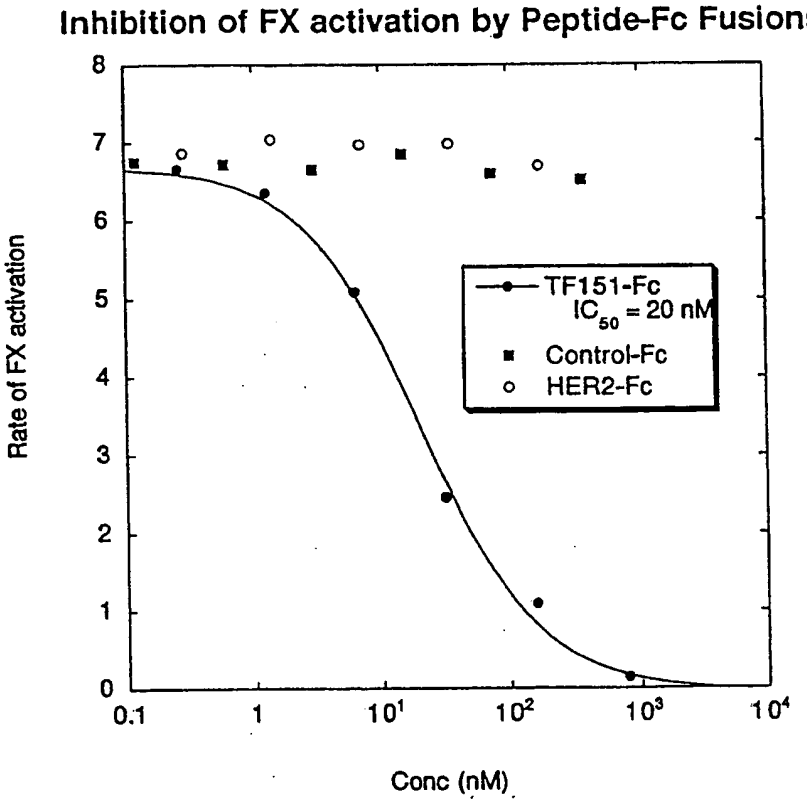
Concentration (nM)
TF151
Control-Fc
TF151-Fc

Figure 6


Inhibition of FX activation by Peptide-Fc Fusions
Rate of FX activation
Conc (nM)
TF151-Fc
IC50 = 20 nM
Control-Fc
HER2-Fc
0
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10^1
10^2
10^3
10^4

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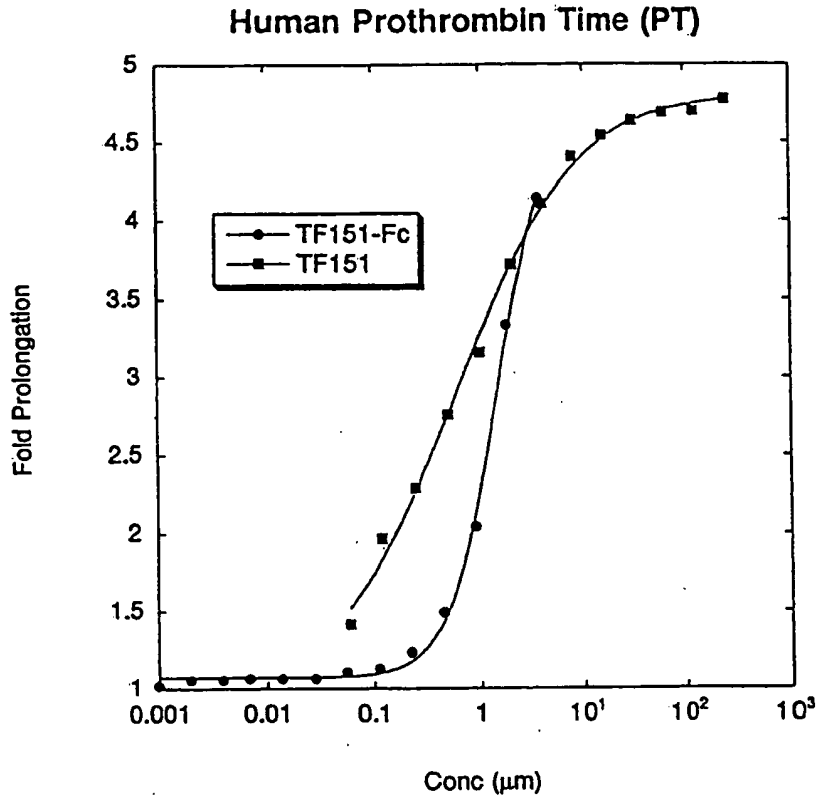
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Fold Prolongation
Conc (µm)
TF151-Fc
TF151
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2
1.5
1
0.001
0.01
0.1
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10¹
10²
10³

Figure 8

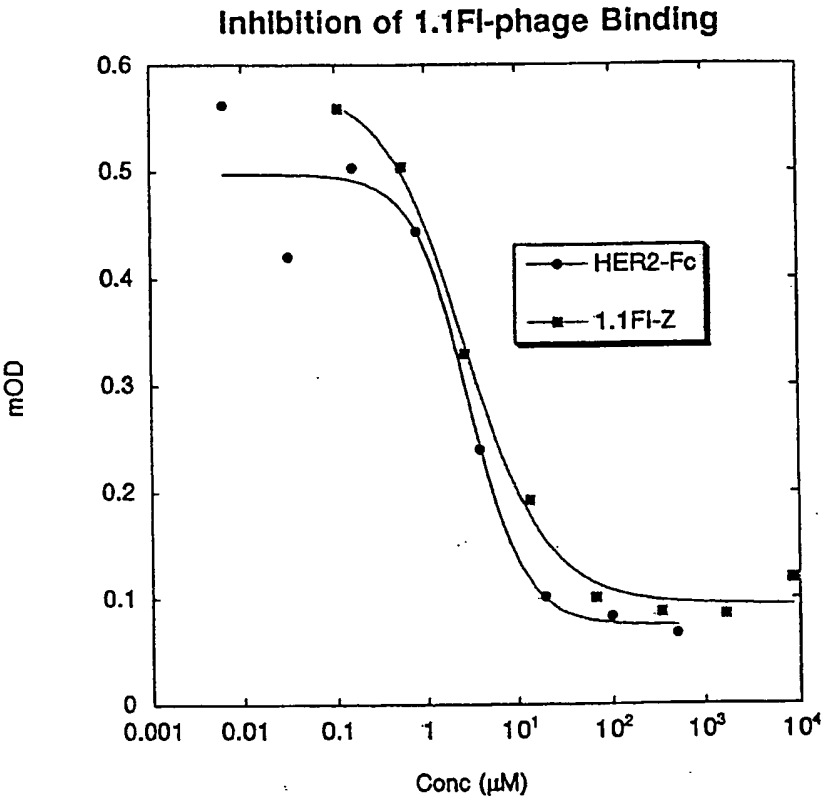
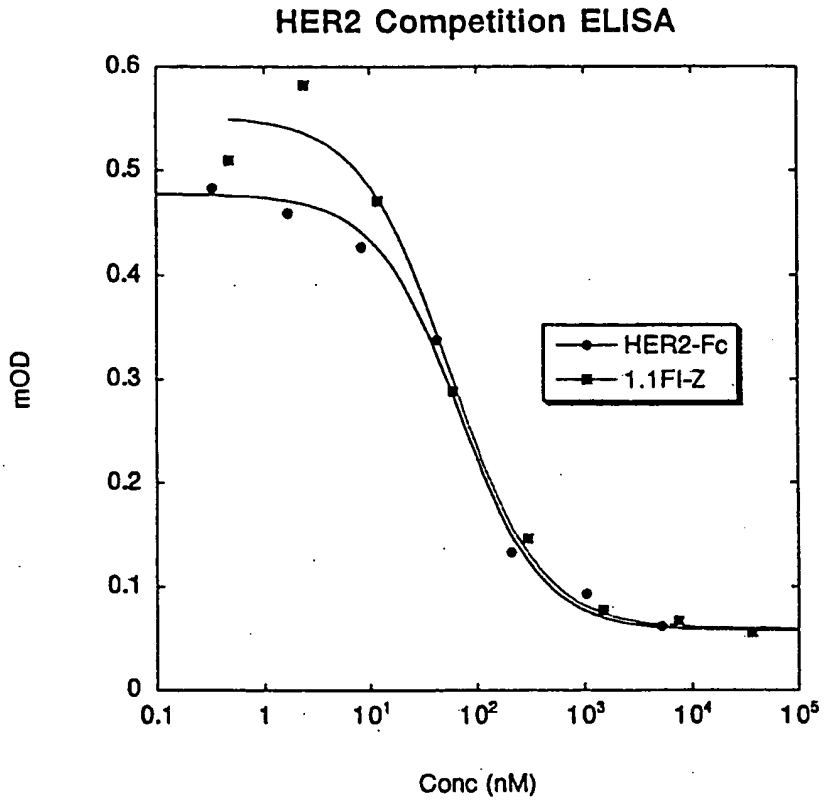

Inhibition of 1.1Fl-phage Binding
mOD
0.6
0.5
0.4
0.3
0.2
0.1
0
0.001
0.01
0.1
1
10¹
10²
10³
10⁴
Conc (μM)
HER2-Fc
1.1Fl-Z

Figure 9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mOD
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0.3
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Conc (nM)
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Sequence Listing

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Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys
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Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
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Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys
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Ile Met His Gln Asp Cys Leu Asn Gly Lys Glu Phe Lys Cys Arg
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Lys Cys Lys Val Asn Asn Lys Asp Leu Pro Ala Pro Ile Glu Arg
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His Leu Pro Ile Gln His Gln Asp Trp Met Ser Gly Lys Glu Phe
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Lys Cys Lys Val Asn Asn Lys Asp Leu Pro Ser Pro Ile Glu Arg
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Ala Pro Val Leu Asp Ser Asp Gly Ser Tyr Phe Ile Tyr Ser Lys
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Leu Asn Met Lys Thr Ser Lys Trp Glu Lys Thr Asp Ser Phe Ser
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Leu Thr Val Asp Thr Asp Ser Trp Leu Gln Gly Glu Ile Phe Thr
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INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 00/18185

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K19/00 C12N15/62 C12N15/86 C12N5/10 C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data, MEDLINE, LIFESCIENCES, AIDSLINE, CANCERLIT, EMBASE, SCISEARCH, WPI Data, EPO-Internal, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 18318 A (UNIV NORTH CAROLINA) 18 August 1994 (1994-08-18) page 20, line 16 -page 21, line 4 page 48, line 7 -page 51, line 11 figure 1C claims 1,9	1-16
X	DE KRUIF J ET AL: "Leucine zipper dimerized bivalent and bispecific SCFV antibodies from a phage display library" IMMUNOTECHNOLOGY,NL;ELSEVIER SCIENCE PUBLISHERS BV, vol. 2, no. 4, 1 November 1996 (1996-11-01), pages 298-299, XP004063238 ISSN: 1380-2933 the whole document	1,8-16

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 November 2000	30/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Covone, M

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INTERNATIONAL SEARCH REPORT

Intern ·al Application No
PCT/US 00/18185

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 750 375 A (BELL LILLIAN ANNE ET AL) 12 May 1998 (1998-05-12) claims page 3, line 16-59	1,11-16
A	WO 94 04689 A (US HEALTH) 3 March 1994 (1994-03-03) page 3, line 35 -page 4, line 15 claims	17-25
A	SALMON S E ET AL: "One bead, one chemical compound: use of the selectide process for anticancer drug discovery." ACTA ONCOLOGICA, (1994) 33 (2) 127-31. , XP000953484 abstract page 129, right-hand column, paragraph 3 -page 130, left-hand column, paragraph 2 page 130, right-hand column, paragraph 3 -page 131, left-hand column, paragraph 1	1-10
A	SCHIER R ET AL: "Isolation of high-affinity monomeric human anti-c- erbB - 2 single chain Fv using affinity-driven selection." JOURNAL OF MOLECULAR BIOLOGY, (1996 JAN 12) 255 (1) 28-43. , XP000953467 abstract page 29, right-hand column, paragraph 3 page 37, left-hand column, paragraph 1	1-8
A	LEE G F ET AL: "A novel soluble tissue factor variant with an altered factor VIIa binding interface." JOURNAL OF BIOLOGICAL CHEMISTRY, (1998 FEB 13) 273 (7) 4149-54. , XP002153113 abstract page 4150, left-hand column, paragraph 2 page 4149, right-hand column, line 37-41	1-10
A	LO K-M ET AL: "High level expression and secretion of Fc-X fusion proteins in mammalian cells" PROTEIN ENGINEERING,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 11, no. 6, June 1998 (1998-06), pages 495-500, XP002125745 ISSN: 0269-2139 the whole document	1-10

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3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/US 00/18185

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
T	DONATE F ET AL: "Dimerization of tissue factor supports solution-phase autoactivation of factor VII without influencing proteolytic activation of factor X." BIOCHEMISTRY, (2000 SEP 19) 39 (37) 11467-76. , XP000960689 abstract page 11468, right-hand column, paragraph 2	1-10

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-25 (all partially)

Present claims 1-10 relate to an extremely large number of possible products having the characteristics as defined in claim 1. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the products claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity, particularly "peptide ligand" lacks any structural features (Article 6 PCT). Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported, disclosed and clear, namely those parts relating to the products disclosed in example 1, disclosing TF151 (seq.ID20) and 1.1FI (seq.ID16) and related Fc fusion polypeptides. Further the search has been carried on what appears to be the concept of the application, namely a peptide ligand bound to an immunoglobulin constant region multimerization domain.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

. ormation on patent family members

Inter ·al Application No
PCT/US 00/18185

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9418318 A	18-08-1994	US 5498538 A	12-03-1996
		US 5747334 A	05-05-1998
		CA 2155185 A	18-08-1994
		EP 0689590 A	03-01-1996
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